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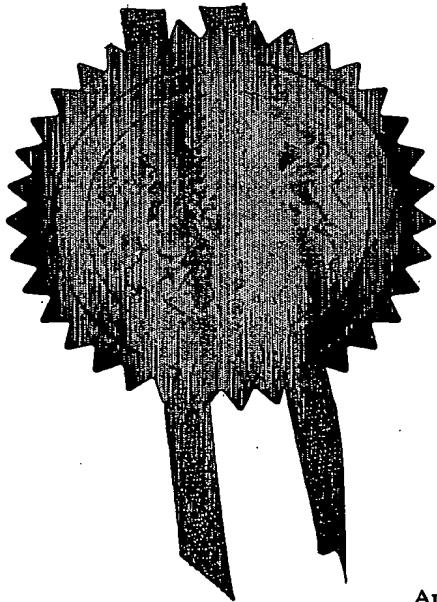
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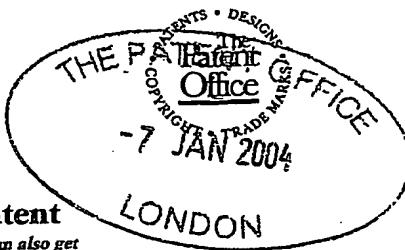


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## COMPLEXES

### **Introduction**

The present invention relates to complexes having adjuvant properties.

5

### **Background to the invention**

Protective immunity against exposure to pathogens is achieved by the integration of two distinct arms of the immune response. They are the innate response and the antigen-specific, also called adaptive, response. The innate immune response 10 acts early after infection, within minutes, by detecting and responding to broad cues from invading pathogens. By contrast, the adaptive immune response takes time, up to two weeks, to become effective, but it provides the fine antigenic specificity that is required for complete elimination of the pathogen and for the generation of immunological memory. Antigen independent recognition of 15 pathogens by the innate immune system leads to the immediate mobilisation of immune effector and regulatory mechanisms which provide the host with three important survival advantages:-

- (i) Rapid initiation of immune responses, both innate and adaptive, and the 20 creation of the inflammatory and co-stimulatory environment required for antigen recognition.
- (ii) Establishment of a first line of defence to hold the pathogen in check during the maturation of the adaptive response.
- (iii) Steering of the adaptive immune response towards the cellular or humoral 25 elements of the immune response that are most effective for protection against a particular infectious agent.

So, although the overall objective of vaccination is the activation of antigen specific immunity, vaccines cannot achieve this goal optimally without first and 30 effectively activating the pathogen detection mechanisms of the innate immune response.

Many vaccines comprise antigens and adjuvants. They are broadly defined by their functional ability to enhance the *in vivo* immunogenicity of the antigens with which they are co-administered. As such, adjuvants represent important

- 5 components of most of the successful vaccines, particularly vaccines based on sub-units of pathogens including isolated fractions of the killed pathogens and/or recombinant antigens. However, as a consequence of the growing appreciation of innate immune mechanisms, and the details of antigen processing and presentation, new and more sophisticated adjuvants are required. Therefore,
- 10 10 traditional and novel adjuvants are now being increasingly separated into those that act as a delivery system and those that act as immune potentiators. Whereas the main function of a delivery system is to localise the vaccine components to antigen presenting cells, immune potentiators directly activate antigen presenting cells through specific receptors that include Toll-like receptors.

15

It is important to keep in mind that infectious agents and most licensed vaccines, particularly live attenuated and killed whole cell products, contain all of the components necessary for activating an integrated immune response. That is because the pathogens used in such vaccines possess all of the relevant antigens

- 20 in a particulate form, as a whole cell, which also contains many potent immune modulators; i.e., pathogen associated molecular patterns. However, the trend in vaccine development is to move away from such products towards safer and better defined sub-unit vaccines that are produced as highly purified recombinant proteins. Unfortunately, such recombinant antigens are often poorly immunogenic
- 25 25 because they lack intrinsic immune potentiating activity. The challenge in sub-unit vaccine development is therefore to reintroduce selective signals for activation of the innate immune response that would be sufficient to mimic natural infection or whole pathogen cell vaccination approaches. The delivery systems and the immune potentiators used to improve the potency of subunit vaccines should be
- 30 30 low cost, more effective and safer than whole cell vaccines.

Although the terms "delivery-system" and "adjuvant" have generally been used interchangeably in relation to vaccines, a clear distinction can often be made and

the respective role of each clearly defined. Included in lists of vaccine "adjuvants" are a number of particulate delivery systems, e.g., emulsions, liposomes, iscoms, virus like particles and microparticles, whose principle mode of action is to promote the delivery of antigens into the key antigen presenting cells responsible for the induction of immune responses. However, they are poor immune modulators and their potency therefore needs to be improved significantly by the addition of an immune potentiator. Unless specified otherwise, the term "adjuvant" is used herein to denote an immune potentiator. The main hurdle to the development of new and improved adjuvants as immune potentiators has been safety because new vaccines must have a minimal number of adverse effects if they are to be acceptable for clinical use. As a result, although many adjuvants have been extensively evaluated pre-clinically and clinically, only aluminium sources, generically called "alum", have been successfully licensed for use as a vaccine adjuvant in North America. Alum is poor at generating cytotoxic CD8 T cell immunity. It preferentially induces a Th2 biased immune response rather than a Th1 biased immune response.

Although proof of concept has been established in animals for the use of many natural pathogen-associated molecular pattern molecules as immune potentiator adjuvants, the trend for the future is to design synthetic analogues. This is mainly due to the lower manufacturing costs and the smaller regulatory hurdles associated with well defined and standardised synthetic immune potentiators.

It was shown as early as 1968 that several synthetic polyanionic polymers protect mice challenged with a lethal dose of Meningo virus and that murine peritoneal macrophages produced factors that inhibited vesicular stomatis virus when exposed to carboxylate co-polymer or a salt thereofs *in vitro* (Merigan & Finkelstein. Interferon stimulating and *in vivo* antiviral effects of various synthetic anionic polymers. Virology 1968; 35: 363-374).

More recently, alginates (polymers isolated from marine algae) have been shown to elicit the release of TNF- $\alpha$ , IL-1 and IL-6 (Otterlei M et ál. Induction of cytokine production from human monocytes stimulated with alginate. J. Immunother. 1991;

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10: 286-291). The 1-4- $\beta$ -mannuronic acid moieties of alginates also induced the release of TNF- $\alpha$  through a CD14/Toll-like receptor 4 mediated mechanism from monocytes. Lignin derivatives and fucoidan also illicit TNF- $\alpha$  release from

5 macrophages (Sorimachi K et al. Secretion of TNF- $\alpha$  from macrophages following

induction with a lignin derivative. Cell Biol. Int. 1995; 19: 833-838; Heinzelmann et al. Modulation of LPS-induced monocyte activation by heparin-binding protein and fucoidan. Infect. Immun. 1998; 66: 5842-5847). Sulphated fucans and dextran

sulphate have also been shown to dramatically increase circulating levels of IL-6,

IL-8, MCP-1, M-CSF and G-CSF when injected into pig-tailed monkeys (Sweeney

10 EA et al. Mobilization of stem/progenitor cells by sulphated polysaccharides does

not require selectin presence. Proc. Natl. Acad. Sci. 2000; 97: 6544-6549).

Chemokines are a heterogenous group of inducible proinflammatory

15 chemoattractive cytokines that include TNF- $\alpha$ , IL-1 and IL-6.  $\beta$ -Chemokines,

which include MIP-1 $\alpha$  and MIP-1 $\beta$ , act as chemoattractants for monocytes,

eosinophils, basophils and lymphocytes (Luster AD. Chemokines - chemotactic

cytokines that mediate inflammation. New Eng. J. Med. 1998; 338:436-446).

Binding of chemokines to their respective receptors leads to a cascade of cellular activation including the generation of inositol triphosphate, the release of

20 intracellular calcium, and the activation of protein kinase C. Hence, chemokines

activate the cellular mechanisms that control chemotaxis. As such, they play a

pivotal role in the recruitment of leucocytes to the areas in which they accumulate

and in the generation of local immune responses.

25 However, excessive release of pro-inflammatory cytokines by the polymers

mentioned above has meant that they are far too toxic to be safely administered to

man. Therefore, although many polymers have been shown to have immuno-

modulatory properties, they have never been effectively exploited for therapeutic

use in man because of the problems associated with their isolation from natural

30 sources, with the reproducibility of isolation, with the synthesis of synthetic

polymers in the laboratory, and with the toxicity of polymers when they are

administered to animals.

**Summary of the invention**

The present invention provides a complex that comprises a narrow molecular weight distribution polymer that includes units derived from an acrylic acid or a salt thereof, and

- 5     (i) a substance that has pharmacological activity against a pathogenic organism,  
or  
(ii) a substance that has pharmacological activity against a cancer, or  
(iii) one or more agents selected from antigens and immunogens.
- 10   The present invention also provides a pharmaceutical preparation which  
comprises a complex of the present invention in admixture or conjunction with a  
pharmaceutically suitable carrier.

The present invention further provides a complex of the invention for use as a  
15 medicament.

The invention also provides a complex for use in treatment of an infection by a  
pathogenic organism and/or for inducing an immune response to the pathogenic  
organism, which complex comprises a narrow molecular weight distribution  
20 polymer that includes units derived from an acrylic acid or a salt thereof, and a  
substance that has pharmacological activity against the pathogenic organism.

The invention further provides a method of treating an infection by a pathogenic  
organism in a subject in need of such treatment, which comprises administering to  
25 the subject a therapeutically effective amount of a complex comprising a narrow  
molecular weight distribution polymer that includes units derived from an acrylic  
acid or a salt thereof, and a substance that has pharmacological activity against  
the pathogenic organism.

30   The invention further provides a method of inducing an immune response to the  
pathogenic organism in a subject in need thereof, which comprises administering  
to the subject an effective amount of a complex comprising a narrow molecular  
weight distribution polymer that includes units derived from an acrylic acid or a salt

thereof, and a substance that has pharmacological activity against the pathogenic organism.

5 The invention further provides a complex for use in treatment of a cancer, which complex comprises a narrow molecular weight distribution polymer that includes units derived from an acrylic acid or a salt thereof, and a substance that has pharmacological activity against the cancer.

10 The invention further provides a method of treating a cancer in a subject in need of such treatment, which comprises administering to the subject a therapeutically effective amount of a complex comprising a narrow molecular weight distribution polymer that includes units derived from an acrylic acid or a salt thereof, and a substance that has pharmacological activity against the cancer.

15 The invention further provides a method of inducing an immune response to a cancer in a subject in need thereof, which comprises administering to the subject an effective amount of a complex comprising a narrow molecular weight distribution polymer that includes units derived from an acrylic acid or a salt thereof, and a substance that has pharmacological activity against the cancer.

20 The invention further provides a complex that comprises a narrow molecular weight distribution polymer that includes units derived from an acrylic acid or a salt thereof, and one or more agents selected from antigens and immunogens, for inducing an immune response to the antigen or immunogen.

25 The invention further provides a method of inducing an immune response to an antigen or immunogen in a subject, which comprises administering to the subject an effective amount of a complex comprising a narrow molecular weight distribution polymer that includes units derived from an acrylic acid or a salt thereof, and the antigens or immunogen,

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The invention further provides a narrow molecular weight distribution polymer that includes units derived from an acrylic acid or a salt thereof, for use with a

substance that has pharmacological activity against a pathogenic organism for the treatment of an infection by the pathogenic organism and/or for inducing an immune response to the pathogenic organism.

- 5     The invention further provides a method of treating an infection by a pathogenic organism in a subject in need of such treatment, which comprises administering to the subject therapeutically effective amounts of a narrow molecular weight distribution polymer that includes units derived from an acrylic acid or a salt thereof, and a substance that has pharmacological activity against the pathogenic  
10    organism.

The invention further provides a method of inducing an immune response to the pathogenic organism in a subject in need thereof, which comprises administering to the subject effective amounts of a narrow molecular weight distribution polymer that includes units derived from an acrylic acid or a salt thereof, and a substance that has pharmacological activity against the pathogenic organism.  
15

The invention further provides a narrow molecular weight distribution polymer that includes units derived from an acrylic acid or a salt thereof, for use with one or  
20    more agents selected from antigens and immunogens for inducing an immune response to the antigen or immunogen.

The invention further provides a method of inducing an immune response to an antigen or immunogen in a subject in need of such treatment, which comprises  
25    administering to the subject therapeutically effective amounts of a narrow molecular weight distribution polymer that includes units derived from an acrylic acid or a salt thereof, and the antigen or immunogen.

The invention further provides a narrow molecular weight distribution polymer that  
30    includes units derived from an acrylic acid or a salt thereof, for use with a substance that has pharmacological activity against a cancer for the treatment of the cancer and/or for inducing an immune response to the cancer.

The invention further provides a method of treating a cancer in a subject in need of such treatment, which comprises administering to the subject therapeutically effective amounts of a narrow molecular weight distribution polymer that includes units derived from an acrylic acid or a salt thereof, and a substance that has  
5 pharmacological activity against the cancer.

The invention further provides a method of inducing an immune response to a cancer in a subject in need thereof, which comprises administering to the subject effective amounts of a narrow molecular weight distribution polymer that includes  
10 units derived from an acrylic acid or a salt thereof, and a substance that has pharmacological activity against the cancer.

The invention further provides a narrow molecular weight distribution polymer that includes units derived from an acrylic acid or a salt thereof for use as an immune  
15 potentiating adjuvant in the manufacture of a vaccine.

The invention further provides a narrow molecular weight distribution polymer that includes units derived from an acrylic acid or a salt thereof for use as an immune potentiating adjuvant in the manufacture of a vaccine that comprises an antigen or  
20 immunogen against which an immune response is to be induced.

In a method for the manufacture of a vaccine, the invention further provides the improvement comprising the use of a narrow molecular weight distribution polymer that includes units derived from an acrylic acid or a salt thereof as an immune  
25 potentiating adjuvant.

### Definitions

The term "a narrow molecular weight distribution polymer that includes units derived from an acrylic acid or a salt thereof" as used herein denotes a polymer  
30 including units derived from an acrylic acid or a salt thereof, for example, one of the polymers described herein, for example, a polymer including units derived from methacrylic acid or a salt thereof, for example, a polymethacrylic acid or a salt thereof, for example a sodium salt thereof. The polymer may be either a

homopolymer or copolymer of an acrylic acid, for example, of acrylic acid or methacrylic acid or a salt thereof. The polymer has a narrow molecular weight distribution, especially a polydispersity of 1.7 or less, for example 1.6 or less, for example 1.5 or less, for example 1.4 or less, for example 1.2 or less, for example 5 less than 1.7, for example less than 1.6, for example less than 1.5, for example less than 1.4, for example, less than 1.2. In general, the lower the polydispersibility, the better. Accordingly, a polydispersibility of less than 1.2 is generally preferred. The polymer generally has a molecular weight of 100,000 or 10 less, for example, in the range from 50,000 to 4,000, for example, from 25,000 to 40,000. The polymer generally has a molecular weight of 100,000 or less, for example, less than 100,000, for example, in the range from 50,000 to 4,000, for 15 example, from 25,000 to 40,000.

The term "PMAA-Na" is used herein to denote polymethacrylic acid, sodium salt. 15 Unless stated otherwise, it denotes a polymethacrylic acid, sodium salt prepared as described in Examples A1 to A4.

The term "complex" is used herein to denote an association between a narrow molecular weight distribution polymer that includes units derived from an acrylic acid or a salt thereof and another substance as defined herein, the association 20 between the components being primarily non-covalent, for example, involving any one or more of ionic, electrostatic and van der Waals forces. Although a complex according to the present invention primarily involves non-covalent association between the components, there may nevertheless be some covalent bonding.

25

#### Brief description of the Figures

##### Figure 1:

A 2% v/v solution of human red blood cells was incubated with a solution of PMAA-Na ("drug") in RPMI 1640 media for 1 h and 24 h at 37°C. No significant 30 toxicity was seen with the PMAA-Na up to a concentration of 2,000 µg/ml after 1 h or after 24 hours. The figure shows the results for 3 human donors (A, B and C).

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Figure 2:

A 2% v/v solution of whole human blood (donor D) was incubated with a solution of PMAA-Na ("drug") in RPMI 1640. No significant toxicity was seen with the PMAA-Na using human whole blood up to a concentration of 500 µg/ml after 1 h  
5 or after 6 h or after 24 hours.

Figure 3a:

Monocyte derived macrophages were incubated with media containing PMAA-Na over the concentration range of 0 - 2,000 µg/ml. The cells were incubated for 71 h  
10 prior to the addition of thiazolyl blue (MTT, Sigma 5 mg/ml). PMAA-Na was not toxic to MDMs at the highest concentration of 2,000 µg/ml tested using the MTT assay and the Trypan blue assay.

Figure 3b:

15 Human peritoneal cells were cultured with PMAA-Na over the concentration range of 0 - 2,000 µg/ml. The cells were incubated for 71 h prior to the addition of MTT. PMAA-Na was not toxic to peritoneal macrophages up to 500 µg/ml. Between 500 µg/m and 2,000 µg/ml, a modest amount of toxicity was seen using the MTT assay and the Trypan blue assays.

20

Figure 4:

Human peritoneal cells from 3 donors (A, B and C) were cultured with PMAA-NA (500 µg/ml) and culture supernatants harvested after 36 h were analysed for MIP-1 $\beta$ . All reagents and the PMAA-Na contained <0.06 endotoxin units/ml (EU/ml).

25 There was a significant release of MIP-1 $\beta$  in the presence of PMAA-Na.

Figure 5:

Human peritoneal cells from 2 donors (A and B) were cultured with PMAA-NA (500 µg/ml and 2,000 µg/ml) and culture supernatants harvested after 36 h were analysed  
30 for TNF- $\alpha$ . There was a significant release of TNF- $\alpha$  in the presence of PMAA-Na at both concentrations.

Figure 6:

Human peritoneal cells were cultured with PMAA-NA (500 µg/ml) and culture supernatants harvested after 36 h were analysed for the pro-inflammatory chemokines MIP-1 $\alpha$ , MIP-1 $\beta$  and IL-8, and for the pro-inflammatory cytokines TNF-

5  $\alpha$ , IL-1 $\beta$  and IL-6. The results with cells from up to 3 different human donors (A, B and C) are shown. The release of these chemokines and cytokines from tissue antigen presenting cells was at a level that would promote a pharmacological Th1 response in man but not high enough to cause significant adverse side-effects in man.

10

Figure 7:

Monocyte derived macrophages were cultured with PMAA-Na at concentrations of up to 2,000 µg/ml. No release of MIP-1 $\beta$  was seen. The results with cells from 3 different human donors (A, B and C) are shown. There is therefore a differential 15 immuno-modulatory effect of PMAA-Na on cells of blood monocyte origin compared to cells of macrophage origin from tissue body based compartments.

Figure 8:

Human peritoneal macrophages were cultured with commercially available PMAA-20 Na (Polymer Standards Service, Germany) of a range of MWt's ( $M_n = 1,300$ , 22,100 and 129,000 g/mol) and narrow molecular weight distribution. One of the commercially available PMAA-Na possessed a MWt comparable to our synthetic preparation of PMAA-Na ( $M_n = 22,000$  g/mol). The culture supernatants were harvested after 36 h. There was no release of MIP-1 $\beta$ . Figure 8 shows the 25 individual results from 2 human donors (A and B).

Figure 9:

Human peritoneal macrophages were cultured with commercially available PMAA-30 Na (Polymer Standards Service, Germany) of a range of MWt's ( $M_n = 1,300$ , 22,100 and 129,000 g/mol) and narrow molecular weight distribution. One of the commercially available PMAA-Na possessed a MWt comparable to our synthetic preparation of PMAA-Na ( $M_n = 22,000$  g/mol). The culture supernatants were

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harvested after 36 h. There was no release of TNF- $\alpha$ . Figure 9 shows the individual results from 2 human donors (A and B).

Figures 10 and 11 and 12:

5 The method was as described for Figure 1. The comparison was made between clinical grade amphotericin B and the amphotericin B – PMAA-Na preparation (both called "drug" in the Figure). Stock solutions of the compounds for testing were prepared in MGM. Human red cell lysis was determined after 1 hour (Figure 10), 6 hour (Figure 11) and 24 hour (Figure 12) incubation in 3 different donors (A,  
10 B and C). Each line represents the results from a single human donor. No toxicity was seen with the amphotericin B – PMAA-Na preparation after a 1 hour incubation. After a 6 hour incubation, the toxicity of the amphotericin B – PMAA-Na preparation was considerably less than that of clinical grade amphotericin B. When the incubation time was increased to 24 h, the toxicity of the clinical grade  
15 amphotericin B increased to 100% but there was no further increase in the toxicity of the amphotericin B – PMAA-Na preparation.

Figure 13:

The method was as described for Figure 11. The degree of red cell lysis by the  
20 amphotericin B – PMAA-Na preparation ("drug") was determined after a 1 hour and 24 hour incubation for concentrations up to 1,000  $\mu$ g/ml.

Figure 14:

PBMCs in RPMI medium were cultured with media containing the amphotericin B –  
25 PMAA-Na preparation ("drug") over the concentration range of 0 - 70  $\mu$ g/ml. The cells were incubated for 1 day or 2 days or 6 days prior to the addition of MTT (5 mg/ml). The compound was not toxic to PBMCs at the highest concentration of 70  $\mu$ g/ml tested using the MTT assay and the Trypan blue assay after 1 day of culture, 2 days of culture or after 6 days of culture. Figure 14 shows the individual  
30 results from up to 3 representative human donors. Each line represents a single donor.

Figure 15:

Monocyte derived macrophages (MDM) were cultured with media containing the compounds ("drugs") up to a concentration of 125 µg/ml. The cells were incubated for 2 days or 3 days prior to the addition of MTT. The amphotericin B – PMAA-Na preparation was less toxic than clinical grade amphotericin B at all of the concentrations up to 125 µg/ml tested using the MTT assay and the Trypan blue assay after both 2 days and after 3 days of culture.

Figure 16:

*Leishmania mexicana* promastigotes were used for these experiments. They were maintained in Schneider's Drosophila growth medium (Invitrogen) supplemented with 15% fetal calf serum (heat inactivated at 56°C for 1 hour) and gentamicin (1 mg/100 ml). The 50% lethal dose ( $LD_{50}$ ) of clinical grade amphotericin B for *Leishmania mexicana* promastigotes was 0.14 µg/ml (Figure 16 inset). The 90% lethal dose ( $LD_{90}$ ) of clinical grade amphotericin B for *Leishmania mexicana* promastigotes was 1.49 µg/ml (Figure 16 inset). The results from 3 experiments with the amphotericin B – PMAA-Na preparation are shown in Figure 16. The 50% lethal dose ( $LD_{50}$ ) of the amphotericin B – PMAA-Na preparation for *Leishmania mexicana* promastigotes was 0.10 - 0.19 µg/ml (Figure 16). The 90% lethal dose ( $LD_{90}$ ) of amphotericin B – PMAA-Na preparation for *Leishmania mexicana* promastigotes was 1.02 - 1.49 µg/ml (Figure 16). The activity of the amphotericin B – PMAA-Na preparation against *Leishmania mexicana* promastigotes was similar to that of clinical grade amphotericin B on a weight per weight basis.

Figure 17:

*Leishmania donovania* promastigotes were used for these experiments. They were maintained in Schneider's Growth Media 199 supplemented with 15% fetal calf serum (heat inactivated at 56°C for 1 hour) and gentamicin (1 mg/100 ml). The 50% lethal dose ( $LD_{50}$ ) of clinical grade amphotericin B for *Leishmania donovani* promastigotes was 0.08 µg/ml (Figure 17 inset). The 90% lethal dose ( $LD_{90}$ ) of clinical grade amphotericin B for *Leishmania donovania* promastigotes was 1.91 µg/ml (Figure 17 inset). The results from 3 experiments with the amphotericin B – PMAA-Na preparation are shown in Figure 17. The 50% lethal dose ( $LD_{50}$ ) of

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- amphotericin B – PMAA-Na preparation for *Leishmania donovani* promastigotes was 0.10 – 0.17 µg/ml (Figure 17). The 90% lethal dose ( $LD_{90}$ ) of amphotericin B – PMAA-Na preparation for *Leishmania donovani* promastigotes was 0.98 – 1.28 µg/ml (Figure 17). The activity of the amphotericin B – PMAA-Na preparation against *Leishmania donovani* promastigotes was therefore similar to that of clinical grade amphotericin B on a weight-per-weight basis.

Figures 18 & 19:

- Leishmania mexicana* amastigotes were used to infect human monocyte derived macrophages that were maintained in RPMI 1640 (Invitrogen) supplemented with 10% human serum (heat inactivated at 56°C for 1 hour) and 200 IU/ml penicillin and 200 µg/ml streptomycin. The infection index was calculated as the average number of parasites/cell multiplied by the percentage of infected cells. A 50% inhibition of intracellular *Leishmania mexicana* amastigote growth was achieved with 0.18 – 0.32 µg/ml (Figure 18) of the amphotericin B – PMAA-Na preparation compared to 0.14 µg/ml of clinical grade amphotericin B (Figure 19a) or 0.45 µg/ml of Ambisome (Figure 19b). A 90% inhibition of intracellular *Leishmania mexicana* amastigote growth was achieved using 1.18 – 1.55 µg/ml of the amphotericin B – PMAA-Na preparation compared to 0.95 µg/ml of clinical grade amphotericin B or 3.89 µg/ml of Ambisome.

Figure 20:

- This graph compares the slopes of relative activity of amphotericin B – PMAA-Na preparation and Ambisome (liposomal amphotericin B, Gilead Sciences, Great Abingdon, Cambridge, UK) against intracellular *Leishmania mexicana* amastigotes. It demonstrates that the killing curve for amphotericin B – PMAA-Na preparation is steeper than the killing curve for Ambisome.

Figures 21 & 22:

- Leishmania donovani* amastigotes were used to infect human monocyte derived macrophages that were maintained in RPMI 1640 (Invitrogen) supplemented with 10% human serum (heat inactivated at 56°C for 1 hour) and 200 IU/ml penicillin

and 200 µg/ml streptomycin. The infection index was calculated as the average number of parasites/cell multiplied by the percentage of infected cells. A 50% inhibition of intracellular *Leishmania donovani* amastigote growth was achieved with 0.30 – 0.71 µg/ml (Figure 21) of the amphotericin B – PMAA-Na preparation compared to 0.54 µg/ml of clinical grade amphotericin B (Figure 22a) or 1.96 µg/ml of Ambisome (Figure 22b). A 90% inhibition of intracellular *Leishmania donovani* amastigote growth was achieved using 2.18 – 3.18 µg/ml of the amphotericin B – PMAA-Na preparation compared to 2.31 µg/ml of clinical grade amphotericin B or >8 µg/ml of Ambisome.

10

Figure 23:

Human peritoneal cells were cultured in macrophage growth medium with each compound. All reagents and compounds contained <0.06 endotoxin unit/ml. The culture supernatants were harvested 24 h later. Interferon- $\gamma$  was measured by EIA (R & D systems). The release of the Th1 promoting cytokine, interferon- $\gamma$ , from peritoneal macrophages was significantly higher in the presence of the amphotericin B – PMAA-Na preparation (100 µg/ml) compared to cells only control, clinical grade amphotericin B, commercial PMAA-Na, or the PMAA-Na.

20

Figures 24:

PBMCs were resuspended in RPMI 1640 supplemented with 10% human serum, 200 µg/ml penicillin and 200 IU/ml streptomycin. All reagents and compounds contained <0.06 endotoxin unit/ml as determined using the *Limulus* amebocyte lysate assay (Pyrotell, Associates of Cape Cod, US). The cells ( $10^5$  cells/well) and each compound (tuberculin PPD and tuberculin-PMAA-Na at the concentrations shown) were mixed in duplicate and incubated at 37°C / 5% CO<sub>2</sub> for 4 days. [<sup>3</sup>H]-Thymidine (specific activity 20-30 Ci/mmol; Amersham Biosciences, UK) was added at 1 µCi/well for a further 18 hours. The cells were then harvested and proliferation determined using a liquid scintillation counter. The results were expressed as the mean counts/minute (cpm) ± sem.

Figure 24a shows the results obtained for the PBMC proliferation after 6 days incubation of the cells of donor A with the tuberculin-PMAA-Na preparation.

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Figure 24a shows that there was significantly increased proliferation of T lymphocytes when they were cultured with 1 µg/ml of the tuberculin-PMAA-Na preparation. There was even more proliferation of T lymphocytes when they were cultured with 10 µg/ml of tuberculin-PMAA-Na preparation ( $P = 0.001$ ).

5

Figure 24b shows the results for donor B. There was significantly more T lymphocyte proliferation with 10 µg/ml of the tuberculin-PMAA-Na preparation than with 10 µg/ml of tuberculin antigen ( $P = 0.002$ ). This shows that the tuberculin-PMAA-Na preparation causes significantly more T lymphocyte proliferation than

10 the tuberculin antigen on its own.

Figure 25:

Human PBMCs were isolated and adjusted to  $2 \times 10^5$  cells/well in RPMI 1640 supplemented with 10% human serum, 200 µg/ml penicillin and 200 IU/ml

15 streptomycin. All reagents and the compounds contained <0.06 endotoxin unit/ml. The PBMCs and the compounds (tuberculin and tuberculin-PMAA-Na at the concentrations shown) were mixed in the wells of an ELISpot PVDF-backed microplate coated with the human interferon- $\gamma$  monoclonal antibody (R&D Systems, UK). Unstimulated cells were used as the negative control and

20 recombinant human interferon- $\gamma$  was used in the positive control. The plate was incubated for 24 hours at 37°C / 5% CO<sub>2</sub>. The manufacturer's instructions were then followed to develop the microplate and the number of positive spots for interferon- $\gamma$  counted. Each spot represented a single interferon- $\gamma$  secreting cell.

25 Figure 25 shows the number of cells producing IFN- $\gamma$ . Figure 25 shows that the tuberculin PPD – PMAA-Na preparation was significantly more effective than tuberculin antigen alone in stimulating interferon- $\gamma$  release from primary human T lymphocytes. This increase in interferon- $\gamma$  secretion from T lymphocytes was seen with 50 µg/ml of the tuberculin PPD – PMAA-Na preparation and with 100 µg/ml of

30 the tuberculin PPD – PMAA-Na preparation.

Figure 26:

Figure 26 shows the survival of *Leishmania donovani* amastigotes in mouse liver macrophages after intravenous treatment with clinical grade amphotericin B, Ambiosome and amphotericin B-PMAA-Na, with untreated controls and blank liposomes as controls. The various compounds were used at the concentrations shown in Figure 26. The number of amastigotes/500 liver cells were counted microscopically and the results expressed as a percentage of the untreated control. There was significant killing activity ( $P<0.0001$ ) of amphotericin B – PMAA-Na preparation at 1 mg/kg of *Leishmania donovani* amastigotes compared to the controls. These results show that the amphotericin B – PMAA-Na preparation was effective in this animal model of visceral leishmaniasis.

#### Detailed description of the invention

As disclosed above, the present invention relates to various uses of a narrow molecular weight distribution polymer that includes units derived from an acrylic acid or a salt thereof in treatment of infections by pathogenic organisms, in treatment of cancer, and/or as an immune potentiating adjuvant, and also relates to complexes that comprise a narrow molecular weight distribution polymer that includes units derived from an acrylic acid or a salt thereof and antigens and/or immunogens, or a substance that has pharmacological activity against a pathogenic organism or against a cancer.

The present invention is based on our observation that a narrow molecular weight distribution polymer that includes units derived from an acrylic acid or a salt thereof induced the release of the beta-chemokines MIP-1 $\alpha$  and MIP-1 $\beta$  and of interferon- $\gamma$  from primary human tissue macrophages, i.e., antigen presenting cells without also inducing the release of toxic amounts of pro-inflammatory cytokines. The polymer was not toxic to human cells at high concentrations. The polymer tested was produced according to the methods described herein. Without being bound by the following theory, we consider that the above observations indicate that a narrow molecular weight distribution polymer that includes units derived from an acrylic acid or a salt thereof has the potential to act as a Th1 immune potentiating adjuvant by stimulating cell surface receptors that include Toll-like

receptors, i.e., to act as a substance that enhances the immune stimulating properties of an antigen.

We have demonstrated in practice that a narrow molecular weight distribution  
5 polymer that includes units derived from an acrylic acid or a salt thereof does indeed act as a Th1 immune potentiating adjuvant. A complex comprising an antigen, namely tuberculin purified protein derivative BP, and a methacrylic acid sodium salt homo-polymer (PMAA-Na) produced as described herein caused more T lymphocyte proliferation that did the tuberculin antigen on its own, and also  
10 increased interferon- $\gamma$  secretion from T lymphocytes more than did the tuberculin antigen alone.

A narrow molecular weight distribution polymer that includes units derived from an acrylic acid or a salt thereof may therefore be used as an immune potentiating  
15 adjuvant in conjunction with antigens and/or immunogens as used in conventional vaccines, for example, antigens and immunogens that are derived directly or indirectly from organisms against which therapeutic and/or protective vaccination is desired. Such antigens and immunogens are, for example, antigens and/or immunogens obtained from natural sources, i.e., derived directly from the relevant  
20 organism, and subunit antigens and immunogens, and antigens and immunogens produced by recombinant DNA technology and/or by chemical synthesis, which antigens and immunogens are derived indirectly from the relevant organisms. The polymer may be formulated in vaccine compositions with the appropriate antigen(s) and/or immunogen(s). Carriers and excipients may be present, as may  
25 delivery system adjuvants. The polymer and the antigen and/or immunogen may be used in the form of a complex of the invention, or may be co-administered, see below.

However, not only does a narrow molecular weight distribution polymer that  
30 includes units derived from an acrylic acid or a salt thereof have an immune potentiating effect on co-administered antigens and immunogens, it also has the ability, in the absence of co-administered antigens or immunogens, to promote protective Th1 immune responses against pathogenic organisms, for example, a

pathogenic organism that is predominantly but not exclusively an intracellular organism, in particular against an organism that exists and persists predominantly in cells of macrophage origin and/or in other antigen presenting cells including dendritic cells. This may be achieved by administering the polymer and a substance that has pharmacological activity against a pathogenic organism (called herein a "drug"), for example, a pathogenic organism that is predominantly but not exclusively an intracellular organism, in particular a pathogenic organism that exists and persists in cells of macrophage origin and/or in other antigen presenting cells including dendritic cells, especially a substance that kills or otherwise disrupts such an organism. The killing or disruption of the organism results in the release of antigenic material. The presence of the polymer potentiates the immune response to the antigen because the dendritic cells that are recruited into this altered microenvironment take up and process the antigens released. The dendritic cells then initiate the CD4+ T cell activation that promotes the generation of effector cytotoxic T-lymphocyte responses. Some of these responses will be therapeutically directed against any persisting organisms in the remaining chronically infected cells and also organisms in the environment of such cells. Other effector cytotoxic T cell responses will be able to provide protective vaccine based responses against future re-infection. Pharmacological treatment, therapeutic vaccination and protective vaccination are therefore achieved.

The invention therefore relates to a narrow molecular weight distribution polymer that includes units derived from an acrylic acid or a salt thereof the polymers for use with a substance that has pharmacological activity against a pathogenic organism in treatment of an infection by the pathogenic organism and/or for inducing an immune response to the pathogenic organism. The invention also relates to a method of treating an infection by a pathogenic organism and/or inducing an immune response to the pathogenic organism in a subject in need of such treatment, which comprises administering to the subject a narrow molecular weight distribution polymer that includes units derived from an acrylic acid or a salt thereof and a substance that has pharmacological activity against the pathogenic organism.

- 20 -

The polymer and the pharmacologically active substance are preferably in the form of a complex of the invention, or alternatively they may be co-administered, see below.

- 5 The pathogens against which an immune response is to be induced are primarily those that replicate and/or persist intracellularly, in particular those that replicate and/or persist in tissue based macrophages and other antigen presenting cells, for example, dendritic cells. Such organisms, and diseases and disorders caused by such organisms, include the following:

10

- a) Organisms that cause superficial mycoses, including ringworm; tinea; thrush; Malassezia infections, including pityriasis versicolor, Malassezia folliculitis, seborrhoeic dermatitis and Scybalidium infections; Otomycosis; and Keratomycosis.

15

- b) Candida species that cause invasive and chronic fungal infections, including Candida albicans; Candida tropicalis and Candida glabrata; Aspergillus species, including Aspergillus fumigatus, Aspergillus flavus and Aspergillus niger; Cryptococcus neoformans; Mucormycosis, for example, caused by species of

20 Absidia, Rhizopus and Rhizomucor; Fusarium species; Trichosporon species; Blastomycosis; Sporothrix species; Sporotrichum species; Histoplasmosis, for example, caused by Histoplasma capsulatum var. capsulatum; African histoplasmosis, for example, caused by Histoplasma capsulatum var. duboisii; Blastomycosis, for example, caused by Blastomyces dermatitidis;

25 Coccidioidomycosis, for example, caused by Coccidioides immitis; Paracoccidioidomycosis, for example, caused by Paracoccidioides brasiliensis; and infections caused by Penicillium marneffei.

30 c) Organisms that cause mycobacterial diseases, for example, tuberculosis and leprosy caused by members of the mycobacterial family, for example, Mycobacterium tuberculosis, atypical mycobacteria, and mycobacterium leprae.

- d) Members of the schistosoma family that cause Schistosomiasis, for example, Schistosoma haematobium, Schistosoma mansoni, Schistosoma japonicum, Schistosoma intercalatum, and Schistosoma mekongi.
- 5     e) Organisms that cause typhoid and paratyphoid fevers, for example, members of the salmonella family of serotypes A, B, C and D.
- f) Organisms that cause toxoplasmosis, for example, Toxoplasma gondii.
- 10    g) Organisms that cause Human African Trypanosomiasis, for example, Trypanosoma brucei gambiense or Trypanosoma brucei gambiense.
- h) Organisms that cause American Trypanosomiasis, for example, Trypanosoma cruzi.
- 15    i) Organisms that cause malaria, for example, Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale and Plasmodium malariae.
- j) Organisms that cause HIV and HTLV infections, for example HIV-1 and HIV-2 and HTLV-I and HTLV-II.
- 20    k) Organisms that cause Pneumocystis carinii infections.
- l) Organisms that cause leishmaniasis, for example, the visceral form, e.g., kala azar or the cutaneous form, for example, L. donovani and L. mexicana.
- 25              Pharmacologically active substances (drugs) used to treat such disease and disorders are well known in the art, see for example; Principles and Practice of Infectious Diseases. by Mandell G.L, Bennett J.E., & Dolin R. Fifth Edition.
- 30    Churchill Livingstone. (2000). Manson's Tropical Diseases. by Cook & Zumla. Twenty-first Edition. Saunders. (2003).

For use according to this aspect of the present invention it is not necessary for a treatment to be fully successful, i.e., to cure the subject or to completely eliminate the organism as the immune response to the organism that is being generated will provide an effective "second line" of defence. Any residual organisms will be

- 5     eliminated by the effector cytotoxic T-lymphocyte responses generated because they will be therapeutically directed against any persisting organisms in the remaining chronically infected cells. What is necessary is that some organisms are killed, thereby releasing antigens. One of the advantages of the present invention is that even if the treatment does not completely eliminate the organism, the
- 10    induction of effector cytotoxic T-lymphocyte responses will provide protective vaccine based responses against future re-infection. Pharmacological treatment, therapeutic vaccination and protective vaccination are therefore achieved.

The same considerations apply to cancer. Use of a complex of the invention that

- 15    comprises a narrow molecular weight distribution polymer that includes units derived from an acrylic acid or a salt thereof and a substance that has pharmacological activity against cancer, especially a cytotoxic agent that kills or partially kills otherwise disrupts the transformed, i.e., cancerous cells. The killing or disruption of the cells results in the release of antigenic material some of which
- 20    will be "seen" by macrophages and antigen presenting cells as non-self antigens. The presence of the polymer potentiates the immune response to the antigen(s) because the dendritic cells that are recruited into this altered microenvironment take up and process the antigen(s) released. The dendritic cells then initiate the CD4+ T cell activation that promotes the generation of effector cytotoxic T-
- 25    lymphocyte responses. Some of these responses will be therapeutically directed against other cancer cells and will therefore provide protective vaccine based responses against the recurrence of the cancer and the growth of any remaining micrometastasis. These responses will be greatest for those cancers in which large numbers of macrophages and antigen presenting cells are present. This is
- 30    especially so in lymphomas and leukaemias. Pharmacological treatment, therapeutic vaccination and protective vaccination are therefore achieved.

- As indicated above, it is generally preferable that the polymer, the substance that has pharmacological activity against a pathogenic organism, or a substance that has pharmacological activity against a cancer (both called "drug") are administered in the form of a complex of the invention. Such a complex is taken up by the antigen presenting cells more effectively than the drug alone, and it also ensures that the drug and the polymer are both present at the same time in the same cell so that when the drug kills the organism and the antigens are released, the polymer is ready *in situ* to generate a micro-environment that potentiates Th1 immune responses. However, the polymer and the drug may be co-administered, either in the same pharmaceutical preparation, or in separate preparations. In the latter case, one preparation may be administered before the other, but it is generally preferable to administer the two preparations substantially simultaneously.
- It is also generally preferable to administer the polymer and antigens and/or immunogens in the form of a complex of the invention. However, the polymer and the antigens and/or immunogens may be co-administered, either in the same pharmaceutical preparation, or in separate preparations. In the latter case one preparation may be administered before the other, but it is generally preferable to administer the two preparations substantially simultaneously.
- A complex of the invention comprises a narrow molecular weight distribution polymer that includes units derived from an acrylic acid or a salt thereof and
- (i) a substance that has pharmacological activity against a pathogenic organism,
  - 25 or
  - (ii) a substance that has pharmacological activity against a cancer, or
  - (iii) one or more agents selected from antigens and immunogens.
- Pathogenic organisms, substances that have pharmacological activity against a pathogenic organism, substances that have pharmacological activity against a cancer, and antigens and immunogens are, for example, as described above.

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A complex of the invention, or a narrow molecular weight distribution polymer that includes units derived from an acrylic acid or a salt thereof may be in the form of a pharmaceutical preparation comprising the complex and a pharmaceutically suitable carrier. The preparation may be or be regarded as a conventional

- 5 pharmaceutical preparation or as a vaccine, or both, depending on the components, in particular the nature of the substances (i), (ii) and (iii). If intended for use in inducing an immune response, a delivery system adjuvant, for example, alum, may also be present.
- 10 If a narrow molecular weight distribution polymer that includes units derived from an acrylic acid or a salt thereof is to be co-administered with a substance (i), (ii) or (iii) as defined above instead of being administered in the form of a complex with the substance, the polymer and the substance (i), (ii) or (iii) may be formulated in the same pharmaceutical preparation or they may be formulated in separate
- 15 preparations, in each case in admixture with a pharmaceutically suitable carrier. If in separate preparations, one may be administered before the other, but it may be preferable to administer the two preparations substantially simultaneously.

As stated above, the present invention encompasses both conventional

- 20 pharmaceutical treatment of diseases and disorders caused by pathogenic organisms and of cancer, and the induction of immune responses to such diseases, disorders and cancer. It will be understood that an immune response induced in a subject in need of such is preferably a therapeutic and/or prophylactic immune response. A therapeutic immune response assists in the treatment of the
- 25 disease or disorder. Such a response may be a short term response. A prophylactic immune response provides longer-term protection, for example, against recurrence of the disease or disorder, or subsequent infection or re-infection, or the growth and spread of the micrometastases of cancer. Such responses are often called therapeutic and prophylactic "vaccination".

30

The use of a narrow molecular weight distribution polymer that includes units derived from an acrylic acid or a salt thereof and a substance that has pharmacological activity against a pathogenic organism in treating a disease

caused by the pathogenic organism and, at the same time, inducing an immune response has been illustrated in the case of leishmaniasis. Infection due to Leishmaniasis can be either of the visceral form, e.g., kala azar or of the cutaneous form. Visceral leishmaniasis is a disseminated protozoal infection. For many years, 5 conventional therapy has consisted of pentavalent antimony given once daily by intravenous or intramuscular injection for 28 days. However, since 1990, the failure of large scale antimony treatment in India has led to the introduction of amphotericin B deoxycholate (AmB) as an effective anti-leishmanial agent. Long-term cure rates of 97% are obtained by the intravenous administration of amphotericin B either daily 10 or, more typically, every other day for a total of 15 - 20 infusions at a dose of 0.75 - 1 mg/kg/day. These prolonged courses of treatment are associated with a high hospital burden and substantial costs. Consequently, this often leads to non-compliance or to the abandonment of the treatment regimen because of the associated adverse events that often occur.

15 Lipid based complexes of amphotericin B accumulate in tissue based macrophages. They have been shown to be very effective against a large number of yeasts and fungi that can grow in human macrophages. It has been shown that lipid formulations of amphotericin B have high level efficacy against visceral leishmaniasis 20 and that they result in a greater than 90% cure rate when given for 5 - 10 days. Sundar et al. have shown that a short course, i.e., 5 day of treatment with liposomal amphotericin B (AmBisome; Gilead Sciences) administered daily in infusions of 1.5 mg/kg/day cured 93% of patients. In another follow-up trial, a single total dose infusion of 5 mg/kg of liposomal amphotericin B cured 91% of patients. There were 25 very few adverse events. (Sundar, S et al. Treatment of Indian visceral leishmaniasis with single or daily infusion of low dose liposomal amphotericin B: a randomised trial. Brit. Med. J. 2001; 323: 419-422. Sundar, S. et al. Single-dose liposomal amphotericin B in the treatment of visceral leishmaniasis in India: a multicenter study. Clin. Inf. Dis. 2003; 37; 800-804). Therefore, single dose liposomal 30 amphotericin B treatment can be considered safe and effective for the treatment of visceral leishmaniasis in India. However, even at a single dose the treatment is costly, and a practical disadvantage is that the lipid-based amphotericin B

formulations are not stable during long-term storage, particularly at the high temperatures of the tropics where many of the cases of visceral leishmaniasis occur.

The immune response that promotes healing and parasite clearance in

- 5 leishmaniasis is dominated by an interferon gamma mediated Th-1 response. Although macrophages ingest leishmania efficiently, they are not activated by the ingestion of the organism; consequently pro-inflammatory chemokines, pro-inflammatoty cytokines and interferon- $\gamma$  are not released. In contrast to macrophages, dendritic cells take up leishmania parasites, mature and then  
10 promote the development of cellular immune responses. In animal models, it has been shown that if infected macrophages can be activated, killing of the parasite ensues. Therefore, two different processes, namely, antigen processing and cellular maturation/activation must be combined for an effective cellular vaccine response. As demonstrated by microbes, substances that possess both an  
15 antigenic component and a dendritic cell activation/maturation component can propel dendritic cells towards a Th1 mediated response. Lipid-based amphotericin B formulations have no immuno-modulatory or adjuvant activity.

A complex of amphotericin B and poly(methacrylic acid, sodium salt) (PMAA-Na)

- 20 according to the invention was prepared and tested, as described in detail in the Examples. The complex was shown to have the following unique properties:

- a) It enables the effective organ delivery of amphotericin B to liver, spleen and lymph nodes after intravenous administration. These are the organs primarily  
25 infected by Leishmaniasis in animals and in man.
- b) It enables the effective intracellular delivery of amphotericin B to Leishmania infected macrophages when given intravenously.
- 30 c) The amphotericin B-poly(methacrylic acid, sodium salt) complex accumulates in the intracellular organelles within which the Leishmania amastigotes survive, multiply and persist, and this enhances the intracellular killing of the Leishmania amastigotes.

- d) *In vivo* studies in an animal model of visceral leishmaniasis showed that the anti-leishmanial activities of clinical grade amphotericin B, the commercial liposomal amphotericin B preparation AmBisome, and the amphotericin B-poly(methacrylic acid, sodium salt) preparation were not significantly different from each other. The amphotericin B-poly(methacrylic acid, sodium salt) preparation was as effective as the commercial liposomal amphotericin B preparation AmBisome in the animal model of visceral leishmaniasis.
- 5
- e) The poly(methacrylic acid, sodium salt) is released from the amphotericin B within tissue macrophages and it then promotes the release of  $\beta$ -chemokines and of interferon- $\gamma$ . This generates a local Th1 adjuvant response.
- 10
- f) The local induction of chemokine and cytokine expression facilitates the recruitment and activation of cells of the innate immune system to the site. This includes immature dendritic cells and CD4+ T lymphocytes. They stimulate macrophages to produce more TNF- $\alpha$ , IL-1 $\beta$  and IL-6.
- 15
- g) Activation of the innate immune system induces dendritic cells to mature and migrate from tissue to regional lymph nodes where their newly released antigens are presented. This results in the induction and generation of CD4+ T cell responses and effector CD8+ T cell responses.
- 20
- h) Therefore, the killing of Leishmania within antigen presenting cells and the subsequent availability of Leishmania antigens in the immediate vicinity of a Th1 promoting adjuvant converts the infected macrophage into a cellular vaccine.
- 25
- i) The cellular immune response is significantly enhanced because of the close proximity of the antigen presenting cells, the release of Leishmania antigens by the killing activity of amphotericin B, and the simultaneous promotion of an appropriate and local Th1 cytokine/chemokine environment.
- 30

j) Concurrent cure of the disease and generation of a therapeutic effector cellular immune response i.e. vaccination generates long-term protective immunity against the organism with which the individual was infected.

5     k) Cure of the disease and therapeutic vaccination are therefore achieved simultaneously and without the need to administer an external adjuvant or an external source of Leishmania antigens.

10    l) Single dose treatment is effective, unlike amphotericin B, which typically requires 15-20 doses.

m) The preparation is more stable, especially at the higher ambient temperatures found in the tropics than the commercially available liposomal amphotericin B preparations.

15    n) The toxicity of free amphotericin that is often seen when it is administered intravenously in man is reduced to a minimum when the preparation is used.

20    o) By forming a complex between amphotericin B and the PMAA-Na, chemical derivatisation of the drug is avoided. Consequently, the efficacy of amphotericin B against Leishmania sp. is not changed or reduced. This is particularly relevant to the amino group in the sugar moiety of amphotericin B because this group is important in mediating the pharmacological activity of this drug. The amine on the sugar moiety has previously been used as a conjugation point because of its reactivity (Conover C.D. et al. Utility of poly(ethylene glycol) conjugation to create prodrugs of amphotericin B. Bioconjugate Chem. 2003; 14: 661-666).

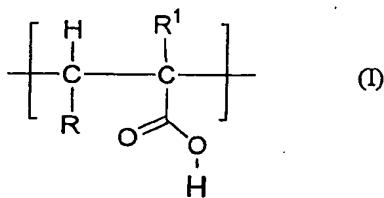
As stated above, the present invention provides a pharmaceutical preparation that comprises a complex of the present invention or a narrow molecular weight distribution polymer that includes units derived from an acrylic acid or a salt thereof in admixture or conjunction with a pharmaceutically suitable carrier.

- A pharmaceutical preparation of the invention may be in a form suitable for administration intravenously, intra-arterially, into the lymphatic circulation, into a lymph node, orally, intraperitoneally, topically, buccally, rectally, to the surface of the skin, transdermally, subcutaneously, intramuscularly, into the joint space,
- 5      intranasally, intravitreally, or pulmonarily, directly to an organ, around an organ, by injection into an organ, or by direct infusion through an organ. The present invention includes administration of a complex of polymer in accordance with the invention by any of such routes. A pharmaceutical preparation of the invention may be in the form of a depot or reservoir preparation, or an aerosol preparation.
- 10     Suitable formulations for that above and other routes of administration are known in the art, see for example, Remington's Pharmaceutical Sciences by E. W. Martin. See also Wang, Y. J. and Hanson, M. A., Journal of Parenteral Science and Technology, Technical Report No. 10, Supp. 42:2S, 1988.
- 15     A complex of the invention or a narrow molecular weight distribution polymer that includes units derived from an acrylic acid or a salt thereof may be "particulate associated". Such particulates may be produced by emulsion, homogenisation and spray drying processes, which are known in the art. For a review of such formulations see for example, Hanes J, Cleland JL & Langer R, Advanced Drug
- 20     Delivery Reviews 28 (1997) 97-119. Pharmaceutical compositions comprising such particulate associated complexes of the invention may be administered mucosally by a pulmonary or nasal route, by ingestion, or by a non-mucosal parenteral route, especially subcutaneously or intramuscularly.
- 25     In a pharmaceutical preparation of the invention in liquid form, the concentration of the polymer may be, for example, from 0.1 to 2,500 µg/ml, for example, from 1 to 500 µg/ml. Other formulations may comprise the polymer in analogous amounts, for example, calculated on the basis of the liquid preparations.
- 30     The present invention is concerned with a narrow molecular weight distribution polymer that includes units derived from an acrylic acid or a salt thereof and with complexes comprising such a polymer and an antigen or immunogen, or a substance that has pharmacological activity against a pathogenic organism or a

- 30 -

- cancer. The polymer may be a homopolymer or copolymer including units derived from an acrylic acid, for example, derived from acrylic acid or methacrylic acid, or a salt thereof. Some polymers including units derived from an acrylic acid are known. As indicated above, the polymer should generally have a narrow
- 5 molecular weight distribution. The polydispersibility is, for example 1.7 or less, for example 1.6 or less, for example 1.5 or less, for example 1.4 or less, for example, 1.2 or less, for example less than 1.7, for example less than 1.6, for example less than 1.5, for example less than 1.4, for example, less than 1.2. In general, the lower the polydispersibility, the better. Accordingly, a polydispersibility of less than
- 10 1.2 is generally preferred. The polymer generally has a molecular weight of 100,000 or less, for example, less than 100,000, for example, in the range from 50,000 to 4,000, for example, from 25,000 to 40,000.

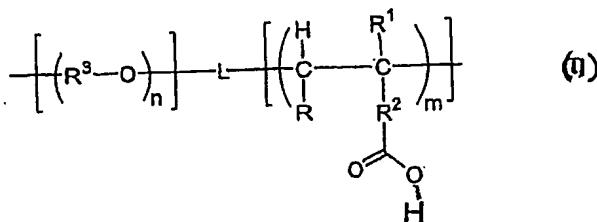
- 15 The polymer including units derived from an acrylic acid or a salt thereof may be a polymer comprising a unit (I)



- 20 wherein R is selected from the group consisting of hydrogen and C<sub>1</sub>-C<sub>18</sub>alkyl, C<sub>2</sub>-C<sub>18</sub> alkenyl, C<sub>7</sub>-C<sub>18</sub>aralkyl, C<sub>7</sub>-C<sub>18</sub>alkaryl, C<sub>6</sub>-C<sub>18</sub>aryl, carboxylic acid, C<sub>2</sub>-C<sub>18</sub>alkoxycarbonyl, C<sub>2</sub>-C<sub>18</sub>alkaminocarbonyl, or any one of C<sub>1</sub>-C<sub>18</sub>alkyl, C<sub>2</sub>-C<sub>18</sub> alkenyl, C<sub>7</sub>-C<sub>18</sub>aralkyl, C<sub>7</sub>-C<sub>18</sub>alkaryl, C<sub>6</sub>-C<sub>18</sub>aryl, carboxylic acid, C<sub>2</sub>-C<sub>18</sub>alkoxycarbonyl, C<sub>2</sub>-C<sub>18</sub>alkaminocarbonyl, substituted with a heteroatom within, or attached to, the carbon backbone; and R<sup>1</sup> is selected from the group consisting of hydrogen and C<sub>1</sub>-C<sub>6</sub>alkyl groups; and salts thereof, for example, alkali metal salts, for example, sodium salts, or ammonium salts thereof.
- 25 Preferably R is selected from the group consisting of hydrogen, C<sub>1</sub>-C<sub>6</sub>alkyl, C<sub>1</sub>-C<sub>6</sub>alkenyl, C<sub>1</sub>-C<sub>6</sub>aralkyl, C<sub>1</sub>-C<sub>6</sub>alkaryl, C<sub>1</sub>-C<sub>6</sub>alkylamido and C<sub>1</sub>-C<sub>6</sub>alkylimido.
- 30 Preferably R is hydrogen or methyl. Preferably R<sup>1</sup> is, independently of R, hydrogen or methyl. In particular R is hydrogen and R<sup>1</sup> is methyl.

Examples of block copolymers that include units derived from an acrylic acid or a salt thereof include block copolymers comprising the unit (II)

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in which R, R<sup>1</sup> and R<sup>2</sup> are defined as above; R<sup>3</sup> is selected from the group  
10 consisting of C<sub>1</sub>-C<sub>18</sub>alkylene, C<sub>2</sub>-C<sub>18</sub>alkenylene, C<sub>7</sub>-C<sub>18</sub>aralkylene, C<sub>7</sub>-  
C<sub>18</sub>alkarylène, and C<sub>6</sub>-C<sub>18</sub>arylene; L is a divalent linker joining the blocks; and m  
and n is each an integer 1 or greater than 1.

Preferably R is selected from the group consisting of hydrogen, C<sub>1</sub>-C<sub>6</sub>  
alkyl, C<sub>1</sub>-C<sub>6</sub> alkenyl, C<sub>1</sub>-C<sub>6</sub> aralkyl and C<sub>1</sub>-C<sub>6</sub> alkaryl, C<sub>2</sub>-C<sub>6</sub> alkoxy carbonyl,  
15 C<sub>2</sub>-C<sub>6</sub> alkaminocarbonyl. Most preferably R is selected from hydrogen and  
methyl.

Preferably R<sup>1</sup> is selected from the group consisting of hydrogen,  
methy, ethyl, propyl, butyl, pentyl or isomers thereof. Most preferably R<sup>1</sup> is  
selected from hydrogen and methyl.

20 Preferably R<sup>2</sup> is selected from a bond or contains at least 1 carbon  
atom or at least 1 heteroatom.

Where R<sup>2</sup> is not a bond, R<sup>2</sup> is connected to CR<sup>1</sup> via a divalent group,  
preferably comprising a carbonyl, C<sub>1</sub>-C<sub>18</sub> alkylene and/or C<sub>6</sub>-C<sub>18</sub> arylenne  
group which may be substituted with 1 or more heteroatoms. Preferably R<sup>2</sup>  
25 comprises a group selected from the group consisting of C<sub>1</sub>-C<sub>6</sub> alkylene, C<sub>6</sub>-  
C<sub>12</sub> arylenne, C<sub>1</sub>-C<sub>12</sub> oxyalkylene and carbonyl-C<sub>1</sub>-C<sub>6</sub> alkylene. Where R<sup>2</sup>  
comprises an alkylene group, it can be branched, linear or cyclical,  
substituted or unsubstituted with one or more alkyl groups, and is preferably  
methylene, 1,2-ethylene, 1,3-propylene, hexylene or octylene. Where R<sup>2</sup>

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comprises an arylene group, preferably it is benzylene, tolylene or xylylene.

Preferably the groups R<sup>3</sup>, which may be the same or different, are selected from the group consisting of C<sub>1</sub>-C<sub>8</sub> alkylene groups, preferably 1,2-alkylene, and C<sub>6</sub>-C<sub>12</sub> arylene groups, most preferably methylene, ethylene,

- 5 1,2-propylene and 1,3-propylene. Preferably all groups R<sup>3</sup> are the same, most preferably all are 1,2-ethylene or 1,2-propylene.

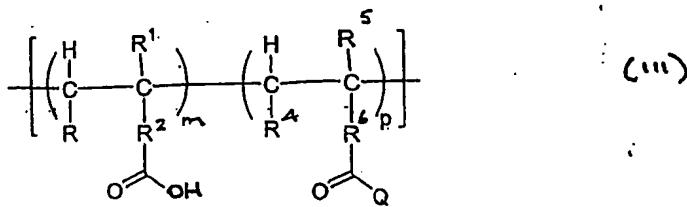
L preferably comprises a C<sub>1</sub>-C<sub>18</sub> alkylene or C<sub>6</sub>-C<sub>18</sub> arylene group which may be substituted and/or interrupted with 1 or more heteroatoms.

- Preferably L comprises a group selected from the group consisting of C<sub>1</sub>-C<sub>6</sub>  
10 alkylene, C<sub>6</sub>-C<sub>12</sub> arylene, C<sub>1</sub>-C<sub>12</sub> oxyalkylene and C<sub>1</sub>-C<sub>6</sub> acyl. Where L  
comprises an alkylene group, it can be branched, linear or cyclical,  
substituted or unsubstituted with one or more alkyl groups, and is preferably  
methylene, 1,2-ethylene, 1,2-propylene, 1,3-propylene, <sup>tert</sup>butylene,  
<sup>sec</sup>butylene, hexylene or octylene. Where L comprises an arylene group, it is  
15 preferably benzylene, tolylene or xylylene. Most preferably L comprises a  
COR<sup>a</sup> group, wherein R<sup>a</sup> is selected from the group consisting of C<sub>1</sub>-C<sub>6</sub>  
alkylene or C<sub>6</sub>-C<sub>12</sub> arylene, preferably methylene, 1,2-ethylene, 1,2-  
propylene, 1,3-propylene, <sup>tert</sup>butylene and <sup>sec</sup>butylene.

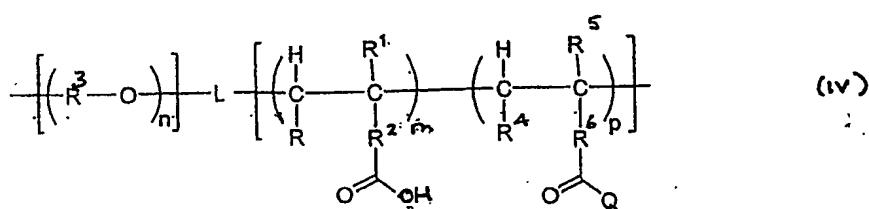
- 20 A polymer may be a homopolymer incorporating unit (I) or may be a copolymer or  
block copolymer incorporating other polymeric, oligomeric or monomeric units, for  
example, a block copolymer comprising units (II) as described above. For  
example, further polymeric units incorporated in a homopolymer or copolymer or  
block copolymer may comprise acrylic polymers, alkylene polymers, urethane  
25 polymers, amide polymers, polypeptides, polysaccharides and ester polymers.  
Preferably, where the polymer is a heteropolymer, additional polymeric  
components comprise polyethylene glycol, polyacrylic acid or polyesters.

- For example, a polymer having units (I) or (II) may also comprise further units  
30 that enable, for example, solubilisation of the precursor polymer and/or that enable  
control of the number of free acrylate acid groups (and hence salt-forming groups)  
in the polymer to be used according to the invention. For example, a polymer  
comprising units (I) may have the structure (III) or (IV)

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in which R, R<sup>1</sup>, R<sup>2</sup> and R<sup>3</sup>, L, m and n are defined as above, R<sup>4</sup>, R<sup>5</sup> and R<sup>6</sup> are selected, independently, from the same groups as R, R<sup>1</sup> and R<sup>2</sup>, respectively; Q denotes a group that is not cleaved or is not substantially cleaved under the conditions used to produce the polymer; and p denotes an integer 1 or greater than 1. If desired, Q may be a targeting group, i.e. a group that targets the polymer to a cell type, eg macrophages, or to an organ eg the liver.

20

Q may be, for example, selected from the group consisting of C<sub>1</sub>-C<sub>12</sub> alkyl, C<sub>2</sub>-C<sub>12</sub> alkenyl, C<sub>7</sub>-C<sub>12</sub> aralkyl, C<sub>7</sub>-C<sub>12</sub> alkaryl, C<sub>1</sub>-C<sub>12</sub> alkoxy, C<sub>1</sub>-C<sub>12</sub> hydroxyalkyl, C<sub>1</sub>-C<sub>12</sub> alkylamino, C<sub>1</sub>-C<sub>12</sub> alkanoyl, C<sub>1</sub>-C<sub>12</sub> aminoalkyl or any one of C<sub>1</sub>-C<sub>12</sub> alkyl, C<sub>2</sub>-C<sub>12</sub> alkenyl, C<sub>7</sub>-C<sub>12</sub> aralkyl, C<sub>7</sub>-C<sub>12</sub> alkaryl, C<sub>1</sub>-C<sub>12</sub> alkocxy, C<sub>1</sub>-C<sub>12</sub> hydroxyalkyl, C<sub>1</sub>-C<sub>12</sub> alkylamino, C<sub>1</sub>-C<sub>12</sub> alkanoyl substituted with an amine, hydroxyl or thiol group. Preferably Q comprises an amine group, for example, a C<sub>1</sub>-C<sub>12</sub> hydroxyalkylamino group, for example, a 2-hydroxypropylamino or a hydroxyethylamino moiety.

The group Q may be a solubilising group for the polymer in aqueous solutions.

30 For example, the polymer may be a water soluble polyacrylamide homo- or copolymer, preferably a polymethacrylamide or polyethacrylaide homo- or copolymer. Furthermore, when the groups Q are not cleaved (or the majority of groups Q are not cleaved) under conditions under which the polymer is produced,

the presence of the groups Q enables control of the number of free acrylate acid groups (and hence salt-forming groups) in the polymer. For example, the proportion of the Q-containing units relative to the number of leaving group X-containing units in the precursor polymer may be selected. The relative proportion 5 of the Q-containing units relative to the number of leaving group X-containing units of the will determine the number of acrylate acid or salt groups in the polymer product. As set out below, the polyanionic nature of the polymer may influence its biological activity. The ability to manipulate the ionic nature of the polymer is therefore a useful in producing a polymer having desired properties.

10 A polymer for use according to the present invention, for example, for forming a complex of the invention, may be, for example, a polymethacrylic acid, for example, a polymethacrylic acid having a polydispersibility of 1.7 or less, for example 1.6 or less, for example 1.5 or less, for example 1.4 or less, for example 15 1.2 or less, for example less than 1.7, for example less than 1.6, for example less than 1.5, for example less than 1.4, for example, less than 1.2. In general, the lower the polydispersibility, the better. Accordingly, a polydispersibility of less than 1.2 is generally preferred. The molecular weight may be 100,000 or less, for example, in the range of from 50,000 to 4,000, for example, from 25,000 to 20 40,000. Examples of such polymethacrylic acids and salts thereof and of their production are given in the Examples herein. Such polymethacrylic acids and salts thereof may be particularly useful in the practice of the present invention.

We have found that the release of  $\beta$ -chemokines and interferon- $\gamma$  from primary 25 tissue macrophages, i.e., antigen-presenting cells induced by the polymethacrylic acid sodium salt (PMAA-Na) complexes produced as described herein was not seen with commercially available samples of methacrylic acid sodium salt homo-polymers having a similar narrow molecular weight distribution. The observed differences in the release of chemokines and cytokines from tissue macrophages 30 suggest that there may be structural differences between PMAA-Na prepared as described herein and commercially available PMAA-Na. Since the  $M_n$  of the PMAA-Na prepared as described herein was comparable to one of the commercial samples

(i.e.,  $M_n \sim 22$  kg/mol), an immunological effect that is due to the influence of molecular weight seems unlikely. PMAA-Na is a polyanion. It has been suggested that the biological effects of polyanions could might be dependent upon structural features such as molecular weight, charge density and tacticity (Ottenbrite, R., et al., *Biological activity of poly(carboxylic acid) polymers.*, in *Polymeric Drugs*, L. Donaruma and O. Vogl, Editors. 1978, Academic Press: New York. p. 263-304.). While not being bound by the hypotheses above and below relating to the biological activity of the polymers used according to the present invention, we consider that the biological data indicates that it may be the preparation method that gave the structural detail that resulted in the PMAA-Na having the unique biological properties described.

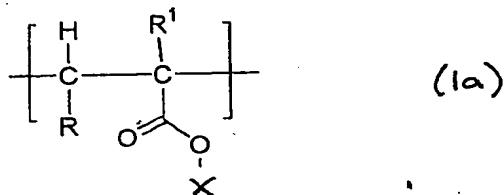
The polymer used in the Examples herein is polymethacrylic acid, sodium salt, which was produced by hydrolysis of poly(N-methacryloxy succinimide) (PMOSu) using sodium hydroxide, for example, under mild conditions. Such a method for the production may be useful for the production of that and other polymers for use according to the present invention, but the invention is not limited to such methods, nor to polymers produced by such methods. The PMOSu used as the precursor polymer was produced as described in WO 01/18080 and in Example A4 herein by homogeneous polymerisation of methacryloxy succinimide using an atom transfer radical polymerisation method, in particular a copper mediated method. Such a method for the production may be useful for the production of that and other precursor polymers for use according to the present invention, but the invention is not limited to such methods, nor to precursor polymers produced by such methods nor to polymers produced from such precursor polymers.

A polymer including units derived from an acrylic acid or a salt thereof for use according to the invention may be produced by hydrolysis of a corresponding precursor polymer that has, in place of the hydrogen atom of the acrylate carboxylic acid in the units derived from an acrylic acid, a group that can be cleaved by hydrolysis to give the acid. A group that can be cleaved by hydrolysis is, for example, an appropriate leaving group, for example, an electron withdrawing group, for example, an acylating group, which is preferably a

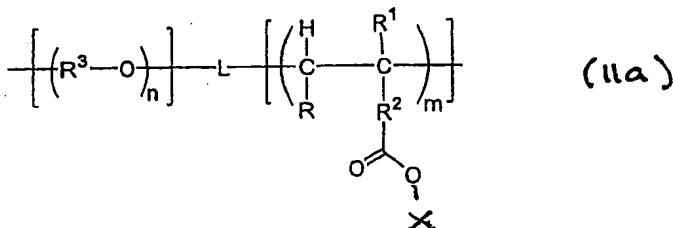
- carboxylate activating, generally selected from the group consisting of N-succinimidyl, pentachlorophenyl, pentafluorophenyl, para-nitrophenyl, dinitrophenyl, N-phthalimido, N-bornyl, cyanomethyl, pyridyl, trichlorotriazine, 5-chloroquinolino, and imidazolyl groups, preferably an N-succinimidyl or imidazolyl group, and especially an N-succinimidyl group. Hydrolysis is preferably carried out using sodium hydroxide, and is preferably carried out under mild conditions, for example, ambient temperature and pressure and gentle heating, for example, 40 to 60°C, for example for 2 to 16 hours.

- 10 A polymer comprising units (I) or (II) as described above may be produced from a polymer (a "precursor polymer") described in WO 01/18080 or from a similar polymer, or from a polymer described in WO 03/059973 or a similar polymer. Such precursor polymers include polymers comprising the unit (Ia) and polymers the unit (IIa)

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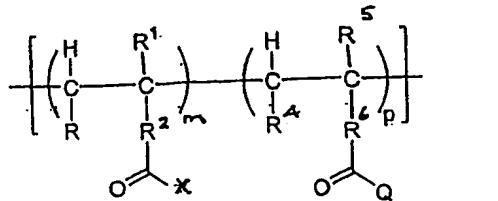


25

in which R, R<sup>1</sup>, R<sup>2</sup>, R<sup>3</sup>, L, m and n are as defined above, and X denotes a leaving group, for example, an acylating group, which is preferably a carboxylate activating, generally selected from the group consisting of N-succinimidyl, pentachlorophenyl, pentafluorophenyl, para-nitrophenyl, dinitrophenyl, N-phthalimido, N-bornyl, cyanomethyl, pyridyl, trichlorotriazine, 5-chloroquinolino, and imidazolyl groups, X preferably denotes an N-succinimidyl or imidazolyl group.

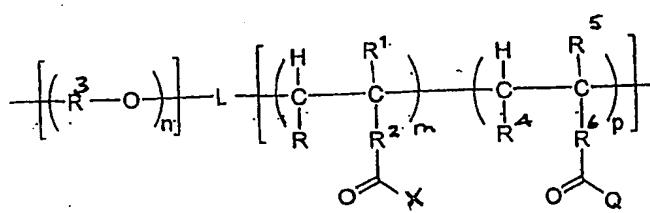
A polymer comprising a unit that comprises the group Q ie a polymer comprising the unit (III) or a polymer comprising the unit (IV) may be produced from a corresponding precursor polymer (IIIa ) or (IVa) in which

5



(IIIa)

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(IVa)

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in which in which R, R<sup>1</sup>, R<sup>2</sup> and R<sup>3</sup>, R<sup>4</sup>, R<sup>5</sup> and R<sup>6</sup>. L. Q. X. m. n and p are defined as above. m, n and p may each represent an integer of up to 500, and preferably the total of m, n and p is not greater than about 500.

A precursor polymer, especially a precursor polymer that comprises a leaving group suitable for cleavage by hydrolysis to give a polymer for use according to the invention may be produced by any appropriate method, in particular any method that enables the production of a narrow molecular weight distribution polymer, especially a polymer having The polymer has a narrow molecular weight distribution, for example 1.7 or less, for example 1.6 or less, for example 1.5 or less, for example 1.4 or less, for example 1.2 or less, for example less than 1.7, for example less than 1.6, for example less than 1.5, for example less than 1.4, for example, less than 1.2. In general, the lower the polydispersibility, the better. Accordingly, a polydispersibility of less than 1.2 is generally preferred. The polymer generally has a molecular weight of less than 100,000, for example, less than 100,000, for example, in the range from 50,000 to 40,000, for example, from 25,000 to 40,000.

- A polymer precursor having the desired polydispersity may be produced by atom transfer radical polymerisation method, for example, a copper mediated atom transfer radical polymerisation, or other methods including free radical
- 5 polymerisation, may be used. Such methods are described in WO 01/18080 and in WO 03/059973. Examples are given below. The invention also provides a polymer including units derived from an acrylic acid or a salt thereof obtainable according to such methods. However, the invention is not limited to polymers produced by such methods. Any method suitable for producing a polymer that
- 10 includes units comprising an acrylic acid atom, in particular having a polydispersity as described above, may be used.

Where the polymerization is carried out by atom transfer radical polymerization, a suitable radical initiator is utilised. Such initiators commonly comprise alkylhalides, preferably alkylbromides. In particular, the 15 initiator is 2-bromo-2-methyl-(2-hydroxyethyl)propanoate. The polymerisation is also carried out in the presence of a polymerisation mediator comprising a Cu(I) complex. Such complexes are usually Cu(I)Br complexes, complexed by a chelating ligand. Typical mediators are Cu(I)Br (Bipy)<sub>2</sub>, Cu(I)Br (Bipy), Cu(I)Br (Pentamethyl diethylene), Cu(I)Br[methyl tris(2-aminoethyl)amine] and Cu(I)Br(N, N, N', N'', N''- 20 pentamethyldiethylenetriamine).

The reaction should take place in the presence of a suitable solvent. Such solvents are generally aprotic solvents, for example tetrahydrofuran, 25 acetonitrile, dimethylformamide, acetone, dimethylsulphoxide, ethyl acetate, methylformamide and sulpholane and mixtures thereof. Alternatively, water may be used. Particularly preferred solvents are dimethylsulphoxide and dimethylformamide and mixtures thereof.

30 As regards the biological properties of a complex of the invention, we have found that different methods of producing a complex of the invention may influence the properties of the complex. It appears to be advantageous to form a complex of the invention by hydrolysing the polymer precursor to form the polymer in the

presence of the antigen or immunogen or the substance that has pharmacological activity against a pathogenic organism or a cancer rather than to form a complex between a preformed polymer and the antigen or immunogen or the substance that has pharmacological activity against a pathogenic organism or a cancer. A

- 5 method of producing a complex of the invention by producing a polymer for use according to the invention, for example, by hydrolysing a polymer precursor, in the presence of the antigen or immunogen or the substance that has pharmacological activity against a pathogenic organism or a cancer of is part of the present invention, as is a complex obtainable by such a method.

- 10 It is considered that, in a complex of the invention, the association between the polymer and the substance that has pharmacological activity against a pathogenic organism or the antigen or the cancer is predominantly non-covalent (i.e., by ionic or Van der Waal's bonding), but that some molecules of the pharmacologically active substance or the antigen may be covalently linked to the polymer. The extent of covalent bonding may be dependent on the nature of the pharmacologically active substance or antigen and on the nature of the polymer.
- 15

- As set out above the term "complex" is used herein to denote an association  
20 between the polymer and the substance that has pharmacological activity against a pathogenic organism or the antigen or the cancer is predominantly non-covalent, but that may include some covalent linkages.

- The complexes of the invention are polyelectrolytes having acidic groups. The extent of deprotonation may vary according to the environment of the complex.  
25 A complex of the invention may be in the form of a salt. A salt may be, for example, with a monovalent, bivalent, trivalent or quadrivalent counter ion. A monovalent counter ion may be, for example, an alkali metal ion, for example, a sodium or potassium ion, or an ammonium ion. A bivalent counter ion may be, for example, an alkaline earth metal ion, for example, a calcium or magnesium ion.  
30 Other counter ions include ions of transition metals, for example, iron, tin etc. More than one type of counter ion may be present and associated with the polymer. Furthermore, the number and proportion of salt-forming groups may be

controlled, for example, by the use of appropriate polymer precursors, for example, as described above.

In one embodiment of the invention, the polymer used in a complex of the

- 5 invention or in conjunction with an antigen or immunogen, or a substance that has pharmacological activity against a pathogenic organism or a cancer is a polymethacrylic acid (PMAA) or a salt thereof, for example, a sodium salt, having a polydispersity of less than 1.4, preferably less than 1.2. The molecular weight of the polymer is preferably 100,000 or less, for example, less than 100,000, for
- 10 example, in the range from 50,000 to 4,000, for example, from 25,000 to 40,000. It may be advantageous to produce the polymer as described or substantially as described in the Examples herein, for example, to hydrolyse a precursor polymer, for example, a precursor polymer having an N-succinimide leaving group, using sodium hydroxide. It may be advantageous to form a complex with an antigen or
- 15 immunogen, or with a substance that has pharmacological activity against a pathogenic organism or a cancer *in situ* during the production of the polymer.

The present invention relates in particular to a complex of the invention that comprises a substance that has pharmacological activity against a pathogenic organism, for example, against leishmaniasis, for example, amphotericin B, to a pharmaceutical preparation comprising such a complex, for example, a pharmaceutical preparation as described above, and to the various uses of such a complex in treating a disease or disorder caused by the pathogen and/or inducing an immune response against the pathogen as described above. The polymer

- 25 component of the complex is a narrow molecular weight distribution polymer that includes units derived from an acrylic acid or a salt thereof, for example, a polymer as described above, for example, a polymethacrylic acid polymer or salt thereof as described immediately above.

- 30 The present invention particularly provides a complex that comprises amphotericin B and a polymethacrylic acid polymer or salt thereof as described immediately above, especially a PMAA polymer or salt thereof produced as described in the Examples herein, and provides a pharmaceutical preparation comprising such a

complex, for example, a pharmaceutical preparation as described above. The invention also provides the various uses of such a complex in treating leishmaniasis and/or inducing an immune response against a pathogen that causes leishmaniasis, including achieving therapeutic and/or prophylactic 5 vaccination against leishmaniasis, as described above.

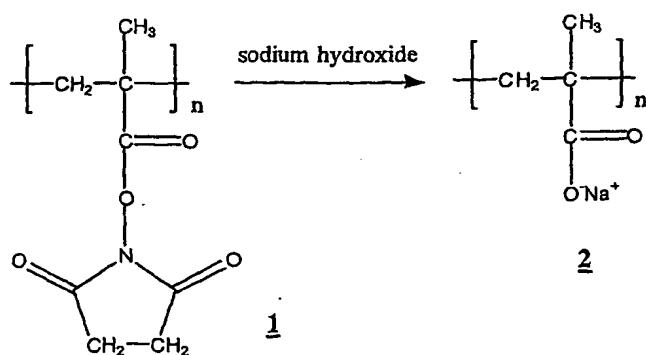
The following non-limiting Examples illustrate the invention.

**Examples:**

- 10 **Example A1: Chemical synthesis: Preparation of methacrylic acid sodium salt homo-polymer:**

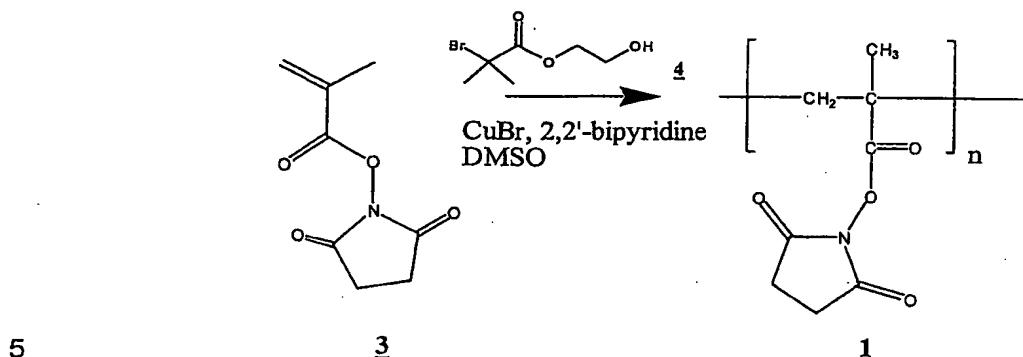
Poly(methacrylic acid, sodium salt) (PMAA-Na) 2 was prepared by the hydrolysis of poly(N-methacryloxsuccinimide) (PMOSu) 1 with sodium hydroxide (Scheme 1).

- 15 The PMOSu 1 had previously been synthesised by the polymerisation of methacryloxsuccinimide 3 (Scheme 2) using an Atom Transfer Radical Polymerisation (ATRP) procedure (Brocchini S. J., and Godwin A., "Uniform molecular weight precursors", International Patent Publication Numbers WO 01/18080A1 & EP 20 99307152.1 (March 15, 2001)).



Scheme 1. Preparation of PMAA-Na 2 from PMOSu 1.

**Example A2: Synthesis of PMOSu 1.**



**Scheme 2. Preparation of PMOSu 1 from methacryloxy succinimide 3.**

- Synthesis of methacryloxy succinimide 3.** To N-hydroxysuccinimide (6.6 g, 57 mmol) in dichloromethane (12 ml) was added dropwise a dichloromethane (12 ml) solution of methacryloyl chloride (6.0 g, 57 mmol) simultaneously with a dichloromethane solution (12 ml) of triethylamine (5.8 g, 57 mmol) maintaining the temperature below 5 °C. After complete addition the reaction mixture was further stirred for 1 h and then washed with aqueous sodium hydrogen carbonate (0.1 M) and water (3 times). The organic phase was then isolated and dried with magnesium sulfate. The solvent was removed to leave the product as a white solid which was recrystallised from ethyl acetate:hexane. Mass 8 g, m.p.= 102°C.  
<sup>1</sup>H, 500MHz, DMSO-d<sub>6</sub>): 2.00 (3H, s, CH<sub>3</sub>), 2.84 (4H, s, (CH<sub>2</sub>)<sub>2</sub>), 6.09 (1H, s, =CH<sub>2</sub>), 6.34 (1H, s, =CH<sub>2</sub>).
- Homogeneous polymerisation:** This reaction is shown in Scheme 2. In a typical copper mediated polymerisation using DMSO as solvent at the preferred weight concentration of 56% in monomer 3, copper(I)bromide (31.3 mg, 0.2 mmol), 2,2'-bipyridine (Bpy) (68.3 mg, 0.4 mmol) and monomer 3 (2.00 g, 10.9 mmol) were added to a round bottomed flask which was then sealed with a septum. Into the flask was then injected DMSO (1.3 g). The resulting brown mixture was gently heated until a solution had formed and then purged with argon for approximately 5 min. An argon purged solution of 2-bromo-2-methyl-(2-hydroxyethyl)propaneate 4

- (46.1 mg, 0.2 mmol) in DMSO (0.2 g) was then injected into the mixture and the flask was heated to 100 °C in an oil bath. The reaction mixture became viscous after a few minutes and was removed from the heat after 10-15 minutes and rapidly cooled. The polymeric product was isolated by addition of 7-8 ml of DMSO
- 5 to dissolve the crude product mixture which was slowly added to a stirred solution of acetone (100 ml) to precipitate PMOSu 1 as a white solid. The acetone solution turned a green colour during the precipitation of polymer 1 due to the dissolution of copper species and the ligand. Atomic absorption analysis indicated the copper content in polymer 1 when at a concentration of 28.0 mg/ml in DMF to be 0.153 ppm. Precipitation of polymer 1 from the DMSO reaction solution into acetone may offer a viable alternative to alumina chromatography that has been typically used in copper mediated polymerisations to remove of copper from the product polymer. The isolated yield of polymer 1 was 1.78 g (89%). The number average molecular weight was 22,700 g/mol and polydispersity index was 1.20. Apparent molecular weights and molecular weight distributions for PMOSu 1 were determined using Waters Styragel HR4 and HR3 (7.3 x 300 mm) columns coupled to a Gibson 133 refractive index detector, poly(methyl methacrylate) PMMA calibration standards and DMF with 0.1% LiCl eluent.
- 10
- 15
- 20 Polymerisations were conducted with different ratios of monomer 3 and initiator 4 to give narrow MWD PMOSu 1 with different molecular weights. These experiments are listed in Table 1 and have been conducted on reaction scales ranging from 2-6 g in methacryloxsuccinimide 3. These homogeneous polymerisation conditions in DMSO gave the polymer 1 in a matter of minutes (e.g. experiment 6 in Table 1 was quenched after 2 minutes to give a significant yield of narrow MWD polymer 1).
- 25
- 30
- Polymerisations were conducted at temperatures ranging from 80-130 °C to maintain solution homogeneity at methacryloxsuccinimide 3 to solvent weight ratios spanning 33-91 %. The preferred solvent was DMSO, but similar results were obtained with DMF. The weight ratio of monomer 3 to polar solvent (DMSO or DMF) was critical for the outcome of the polymerization. In DMSO at weight ratios less than 56% monomer 3 (e.g. 50 and 41%) resulted in lower yields of

- polymer (52 and 40% respectively). At weight concentrations higher than 60% monomer 3 in DMSO, the polymerisation solution solidified. Likewise in DMF, the weight concentration of monomer was critical for the outcome of the polymerisation reaction, however the maximal yield in DMF was less than in DMSO.
- 5 50 % yield of polymer 1 was isolated at monomer 3:DMF weight ratio of 61 %. No polymer was isolated when the reaction was conducted at a monomer 3 weight ratio of 33 %. At higher monomer weight concentrations (above 75 %), the reaction mixture solidified.

10

TABLE 1

Experiment	1:2:CuBr:Bpy <sup>a</sup>	T, °C	Yield, %	$M_n$	$M_w / M_n$
1	10:1:1:2	100	85	12500	1.17
2	20:1:1:2	80	92	16800	1.15
3	50:1:1:2	100	89	22700	1.20
4	100:1:1:2	100	96	29000	1.14
5	150:1:1:2	110	80	40700	1.13
6 <sup>b</sup>	100:1:1:2	100	49	23330	1.15

- (a) Ratio of initial monomer and initiator concentrations.  
15 (b) Reaction stopped after 2.5 minutes by dilution with DMSO and rapid cooling.

(B) *Precipitation polymerisation:* Copper mediated polymerisations of monomer 3 in solvents such as THF, ethyl acetate, toluene and acetone also gave narrow MWD polymer 1. Yields ranged from 10-95 % depending on the polymer molecular weight.

20 Higher molecular weight polymer 1 up to 25,000 g/mol was obtained in mixed solvent systems such as THF and ethylene carbonate. At molecular weights above 10,000 g/mol the yields were sometimes less than that observed when the polymerisation was conducted in DMSO or DMF. Exemplary copper mediated polymerisations using 0.5 g in monomer 1 were conducted in THF over 25 a 16 h time period at 70 °C are listed in Table 2. The copper chelating legend used in these THF reactions was N, N, N', N", N"-pentamethyldiethylenetriamine (PMDETA). Additional precipitation reactions are listed in Table 3 with 2-bromo-2-methyl propionic acid (BMA) used as initiator.

TABLE 2

Experiment	1:2:CuBr:PMDETA <sup>a</sup>	$\bar{M}_n$	$M_w/M_n$
1	100:1:1:1.2	14,800	1.10
2	200:1:1:1.2	1,800	1.12
3	100:1:0.3:1.2	13,100	1.09

5 TABLE 3

[MOSu] <sub>0</sub> :[BMA] <sub>0</sub> : [CuBr] <sub>0</sub> :[Ligand] <sub>0</sub>	Solvent (wt%)	Yield (%)	$M_n$	$M_w/M_n$
50:1:1:2(bipy)	EC (45)	39	34,600	1.43
100:1:1:1.2(PMDETA)	THF/EC 5:1 (74)	67 <sup>a</sup>	25,300	1.39
100:1:1:1(bipy)	THF/EC 6:1 (78)	75 <sup>a</sup>	19,500	1.29
100:1:1:1(bipy)	THF/EC 20:1 (84)	82 <sup>a</sup>	12,200	1.12
50:1:0.5:1(bipy)	THF/EC 20:1 (84)	74 <sup>a</sup>	12,000	1.09
100:1:1:1(bpy)	THF/EC 27:1 (87.5) <sup>b</sup>	22	10,000	1.08
50:1:0.5:1(bpy)	THF/EC 27:1 (87.5) <sup>b</sup>	24	12,500	1.10

<sup>a</sup>The product was directly filtered and washed.

<sup>b</sup>Freshly distilled THF

10 **Example A3: Hydrolysis of PMOSu 1 to give PMAA-Na 2 (Scheme 1).**

PMOSu 1 (1.00 g,  $M_n = 24,800$  g/mol,  $M_w/M_n = 1.20$ , DMF eluent, PMMA standards) was dissolved in DMF (5.0 ml) and then 2 M aqueous sodium hydroxide (6.0 ml) was added drop-wise under stirring causing some precipitation of the polymer. The reaction vessel quickly became warm and a homogeneous solution soon followed. The solution was then heated at 70 °C for 24 h after which time further water was added (approximately 50 ml). The diluted solution was then dialysed against water using regenerated cellulose membrane (MWCO 2000, SpectraPor). Freeze-drying of the dialysed solution gave a white solid product, PMAA-Na 2 (0.3 g). GPC (PBS eluent, PMAA-Na standards)  $M_n = 22,000$  g/mol and  $M_w/M_n = 1.28$ ; FT-IR (ATR) 1675 cm<sup>-1</sup>, 1547 cm<sup>-1</sup>, 1194 cm<sup>-1</sup>.

25 The molecular weight value for PMAA-Na 2 can be used to determine the degree of polymerization (DP) to know the number of repeat units for any polymer derived from 1. In this example the repeat unit molecular weight of PMAA Na 2 is 108, the DP for this sample was approximately 203 (i.e. 22,000 g/mol ÷ 108 g/mol). This means the DP for PMOSu 1 is 203, and since the molecular weight of the repeat

unit of PMOSu 1 is 183 g/mol, then the absolute number average molecular weight of PMOSu 1 in this example was 37,149 g/mol (i.e. 183g/mol  $\times$  203). The value of 203 for the DP of narrow MWD PMOSu 1 can be used in an analogous fashion to determine the absolute molecular weight of polymers derived from 1.

5

***Example A4: Hydrolysis of PMOSu 1.***

PMOSu 1 (200 mg,  $M_n = 32,200$  g/mol,  $M_w/M_n = 1.24$ , DMF eluent, PMMA standards), was dissolved in DMSO (2 ml) and under stirring, 1 M aqueous sodium hydroxide (2.2 ml) was added dropwise. Typically, some precipitation occurred.

- 10 Additional water (23 ml) was added immediately following the addition of sodium hydroxide and the mixture left to stir at room temperature for 1 h. The resulting reaction solution was then diluted to 44 ml and dialysed against 1 L water for 24 h using Visking dialysis membrane (MWCO 7000, Medicell International). During dialysis, the water was changed 6 times. The dialysed solution was filtered  
15 through a 0.2  $\mu$ m filter and then freeze-dried to afford 0.2 g of PMAA-Na product  
2. GPC (Triple detection,  $\text{NaNO}_3$  0.2 M/ 10%  $\text{CH}_3\text{CN}$ )  $M_n = 35,700$  g/mol and  
 $M_w/M_n = 1.18$ .

Each preparation of PMAA-Na 2 that was prepared was characterised using  $^1\text{H}$

- 20 Nuclear Magnetic Resonance and Fourier-Transform Infra-Red Spectroscopy and was found to be consistent with the desired product.

***Example A5: Synthesis of PMOSu 1 by free radical polymerization with AIBN followed by hydrolysis to give PMAA-Na 2.***

- 25 An argon purged solution of methacryloxy succinimide 3 (3 g) and AIBN (0.135 g) in acetone (30.0 ml) was heated at 50°C in a closed vessel for 24 h. The white precipitate formed was isolated by filtration, dissolved in DMSO (6.0 ml) and re-precipitated in rapidly stirring acetone. After isolating by filtration and drying in vacuo, some of the dried PMOSu 1 (0.48 g) in DMSO (4.8 ml) was mixed with 1N  
30 sodium hydroxide (5.2 ml) and water (56 ml). The resulting solution was allowed to stir for 1 h at room temperature whereupon it was diluted to 105 mL with fresh water and dialysed against 5 L of water for 24 h using a Visking dialysis membrane (MWCO 7000, Medicell International). The 5 L of water was changed 6

times. The dialysed solution was filtered through a 0.2 µm filter and then freeze-dried to afford PMAA-Na 2 (0.56 g) as a solid product; Mw 26,100 Da, Mn 15,200 g/mol, Mw/Mn 1.7 (SEC 0.2 M aqueous NaNO<sub>3</sub>/ 10% CH<sub>3</sub>CN, PMAA-Na standards)

5

**Example A6: Synthesis of PMOSu 1 by free radical polymerization with 4,4'-azobis(cyanovaleric acid) followed by hydrolysis to give PMAA-Na 2.**

A pressure tube was charged with methacryloxy succinimide 3 (0.5-1.0 g), and 10 4,4'-azobis(cyanovaleric acid) (15-30 wt%) and solvent (for example acetone, freshly distilled THF, or mixtures of these solvents including toluene and ethylene carbonate) and the resulting solution was sealed and purged with argon for 15 min. The sealed flask was then heated at 70-120 °C for 0.25-2.5 h. See Table 4 for the conditions of example reactions. PMOSu 1 was isolated as a white precipitate by filtration and dried in vacuo and GPC determined in DMF using 15 PMMA standards.

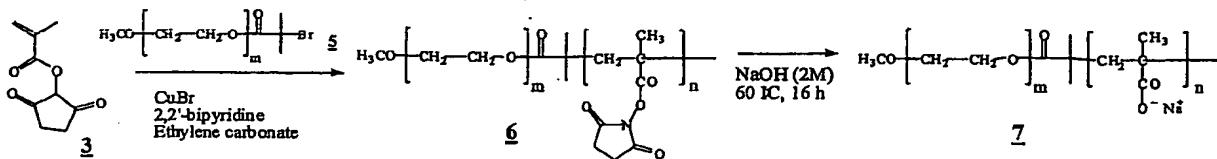
TABLE 4

initiator wt%	Solvent (wt% monomer 3)	Time (h)	T/°C	M <sub>n</sub> (g·mol <sup>-1</sup> )	M <sub>w</sub> /M <sub>n</sub>	Yield (%)
15	Acetone (85)	3.5	70	14,100	1.65	80
15	THF (86)	0.5	70	15,700	1.54	48
30	THF (81.6)	2.5	70	14,500	1.51	74
30	THF (81.6)	2	100	9,200	1.40	46.5
30	Toluene (81.2)	1	100	11,500	1.70	75

20

**Example B: Synthesis block copolymer PEG-PMOSu 6 and its hydrolysis to give block copolymer PEG-PMAA-Na 7.**

The polymerisation and hydrolysis reaction is shown in Scheme 3 and examples have previously been described (Brocchini S. J and Godwin A. "Block 25 Copolymers." International Patent Publication number WO 03/059973 and Pedone et al, An information rich biomedical polymer library, J. Mat. Chem., 2003, 13, 2825-2837).



**Scheme 3. Synthesis of block copolymers PEG-PMOSu 6 and PEG-PMAA-Na**

5 7.

(A) *Preparation of macroinitiators 5.*

The PEG macroinitiators 5 were prepared by the procedure of Jankova et al (Macromolecules (1998), 31, 538-541). Triethylamine ( $12.5 \times 10^{-3}$  mol, 1.265 g, 10 ml) in 15 ml dry  $\text{CH}_2\text{Cl}_2$  was added to a 250 ml three-neck round-bottom flask equipped with a condenser, dropping funnel, gas inlet and a magnetic stirrer. After cooling to 0°C 2,2-bromoisobutryl bromide ( $12.5 \times 10^{-3}$  mol, 2,874 g., 1.55 ml) in 10 ml  $\text{CH}_2\text{Cl}_2$  was added and the mixture purged with nitrogen. Then monomethoxy capped PEG ( $M_n = 2,000$  g/mol) ( $5 \times 10^{-3}$  mol, 10 g) in 50 ml  $\text{CH}_2\text{Cl}_2$  was added dropwise during 1h under nitrogen. The PEG had been previously dried by azeotropic distillation in toluene and the residual toluene removed in vacuum. The temperature of the reaction mixture was allowed to rise to room temperature and the reaction continued for 18h. The solution was filtered, half of the solvent evaporated under vacuum and the product was precipitated in cold ether. The precipitate was recrystallised in absolute ethanol (stored overnight in the fridge). The macroinitiator 5 was filtered, washed with cold ether and dried under vacuum. The crude product was purified by dissolving 4 g in 80 ml water. The solution pH was raised to pH 8 in order to hydrolyse the excess of i-BuBr. Then the solution was extracted with  $\text{CH}_2\text{Cl}_2$  (70 ml). A stable emulsion was obtained and several hours were needed for complete phase separation. The solvent was removed in vacuum. The product was dissolved in hot EtOH and put in a fridge to crystallise. Then it was filtered and washed with ether and dried under vacuum. The purified product 5 was white in colour. The degree of substitution calculated by the H MNR spectra. This procedure was also used to prepare PEG macroinitiators 5 derived from PEG 5000 and PEG 10000.

(B) Representative preparation of block PEG-PMOSu 6.

A mixture of monomer 3 (as synthesised in WO 01/18080), ethylene carboate and bipyridine was placed in a tube sealed with a septum and it was purged with argon for 5 minute and the CuBr was added. The misture was gently heated to form a  
5 solution (deep brown in colour) and purged with argon for another 30 minutes.  
Then a solution of the PEG macroinitiator 5 in the amount relative to the monomer specified in Table 5 in ethylene carbonate (gently heated to liquidfy both the ethylene carbon and 5) was purged with argon for 10 minutes and added to the  
10 monomer solution by syringe washed with argon. The mixture was placed in an oil bath and stirred. The reaction was stopped by exposure to air, cooling and diluting with DMF. Then the solution was passed through a column filled with alumina and the polymer precipitated in methanol. The PEG-PMOSu 6 precipitate was filtered, washed with ether and dried in vacuum. The PEG-PMOSu 6 was obtained as a  
15 white powder. Table 5 show polymerisation conditions, yield and molecular weight characteristics of polymerisation conducted with microinitiator 5 derived from PEG of molecular weight 2000 g/mol.

TABLE 5

	3 mmol (g)	5 (mmol) (g)	CuBr (mmol) (g)	Bpy (mmol) (g)	3:5:CuBr: bpy	EC <sup>1</sup> (g)	T (°C)	Time (h)	Yield (%)	Mn <sup>2</sup> (Mw/Mn)	PD (Mw/Mn)
1	5 0.917	0.1 0.215	0.1 0.0157	0.2 0.0342	50:1:1:2	1.3	80	1.5	56	17,000	1.39
2	5 0.917	0.1 0.215	0.1 0.0157	0.2 0.0342	50:1:1:2	1.0	80	1.0	40	17,600	1.30
3	5 0.917	0.1 0.215	0.1 0.0157	0.2 0.0342	50:1:1:2	1.3	80	5.0	87	20,100	1.53
4	5 0.917	0.05 0.1075	0.05 0.0078	0.1 0.0171	100:1:1:2	0.65	80	5.0	100	31,300	1.32
5	2.5 0.458	0.05 0.1075	0.05 0.008	0.1 0.0171	50:1:1:2	0.65	60	5.0	20	13,230	1.35
6	5 0.917	0.1 0.215	0.1 0.0157	0.2 0.0342	50:1:1:2	1.3	80	5.5	32	19,700	1.29
7	2.5 0.458	0.05 0.1075	0.05 0.005	0.1 0.0171	50:1:1:2	0.65	100	5.0	64	28,000	1.38
8	2.5 0.458	0.5 0.1075	0.05 0.008	0.1 0.0171	50:1:1:2	0.65	80	5.0	56	25,700	1.29
9	2.5 0.458	0.05 0.1075	0.05 0.008	0.1 0.0171	50:1:1:2	0.65	80	5.0	47	22,800	1.27
10	2.5 0.458	0.05 0.1075	0.05 0.008	0.1 0.017	50:1:1:2	0.5	80	5.0	74 <sup>3</sup>	30,200	1.28
11	2.5 0.458	0.05 0.1075	0.025 0.004	0.15 0.017	50:1:0.5:3	0.5	80	3.0	42.4 <sup>3</sup>	18,100	1.25
12	5 0.917	0.05 0.1075	0.05 0.008	0.1 0.0171	100:1:1:2	0.5	110	4:35	100	18,600	1.33

20

<sup>1</sup> EC = ethylene carbonate

<sup>2</sup> Gel permeation chromatography used DMF eluent with PMMA standards

<sup>3</sup> The reaction mixture was purged with argon for 1 hour.

(C) Detailed example of the synthesis of PEG-PMMA-Na 7 derived from poly(ethylene glycol) of molecular weight 2000 g/mol.

Copper(I) bromide (31.2 mg, 0.2 mmol), bpy (68.4 mg, 0.4 mmol) and N-

- 5 methacryloxsuccinimide 3 (3.67 g, 20 mmol) and ethylene carbonate (2 g) were added to a round-bottomed flask that was then sealed with a septum. The resulting brown mixture was gently heated until a solution had formed and then purged with argon for approximately 15 min. An argon-purged solution of PEG<sub>2000</sub>-macroinitiator 5 (430 mg, 0.2 mmol) in ethylene carbonate (0.6 g) was  
10 then injected into the mixture and the flask was heated to 80 °C in an oil bath for 1 h. The viscous reaction mixture was then removed from the heat and rapidly cooled. The resulting crude polymeric product was dissolved by in DMF (8 ml) and the resulting solution was slowly added to stirred methanol (500 ml) to precipitate PEG-PMOSu 6 as a white solid (3.8 g, 92%);  $M_n = 37,160 \text{ g}\cdot\text{mol}^{-1}$   $M_w/M_n = 1.32$   
15 SEC (DMF 0.1% LiCl). <sup>1</sup>H NMR (DMSO-d<sub>6</sub>) δ 1.38 (br; 3H, CH<sub>3</sub>), 2.42 (br m, 2H, CH<sub>2</sub>C), 2.78 (br, 4H, CH<sub>2</sub>CH<sub>2</sub>), 3.50 (s, 4H, OCH<sub>2</sub>CH<sub>2</sub>O).

Hydrolysis of block PEG-PMOSu 6 was conducted. Hydrolysis of this to PEG-

PMMA-Na salt was conducted by slowly adding 2M NaOH (0.6 ml) dropwise to a

- 20 stirred solution of block polymer precursor (0.1 g, 0.54 mmol) in DMF (0.5 ml) in a 10 ml round bottom flask. The reaction mixture was heated to 60 °C for 16 h and more water (5.0 ml) was added. The dilute solution was then dialysed using Visking tubing (MWCO 12,000-14,000) and the dialysate was freeze dried to give as white solid product PEG<sub>2000</sub>-PMMA-Na salt 7 (95% yield)  $M_n = 34,110 \text{ g}\cdot\text{mol}^{-1}$   
25  $M_w/M_n = 1.34$  (Triple detection-SEC; NaNO<sub>3</sub> 0.2 M/ 10% CH<sub>3</sub>CN). <sup>1</sup>H-NMR (D<sub>2</sub>O) δ 0.85-0.96 (br s, 3H, CH<sub>3</sub>) 1.60, 1.88 (br, m, 2H, CH<sub>2</sub>) and 3.58 (s, 4H, OCH<sub>2</sub>CH<sub>2</sub>O) confirming the hindered ester bond to the PEG<sub>2000</sub>-block was not hydrolysed.

(D) Detailed example of the synthesis of PEG-PMMA-Na 7 derived from

30 poly(ethylene glycol) of molecular weight 5000 g/mol.

Copper(I) bromide (10.4 mg, 0.072 mmol), 2,2'-bipyridine (bpy, 22.6 mg, 0.145

mmol), and N-methacryloxsuccinimide (2.0 g, 10.8 mmol), and PEG<sub>5000</sub>-

macroinitiator 5 (362 mg, 0.072 mmol) and ethylene carbonate (2.362 g) were

added to a Schlenk flask which was then sealed with a septum. The resulting brown mixture was degassed by freeze thaw method. The mixture was heated to 110 °C in an oil bath for 2 h. The viscous reaction mixture was then removed from the heat and rapidly cooled. The resulting crude polymeric product was then dissolved in DMF (8 ml). The resulting solution was slowly added to stirred methanol/diethylether (2:1 v/v, 300 ml) to precipitate PEG-PMOSu 6 as a white solid (1.8 g, 76%).  $M_n = 32,860 \text{ g}\cdot\text{mol}^{-1}$   $M_w/M_n = 1.36$  (SEC (DMF 0.1% LiCl)).  $^1\text{H}$  NMR (DMSO-d<sub>6</sub>)  $\delta$  1.38 (br, 3H, CH<sub>3</sub>), 2.42 (br m, 2H, CH<sub>2</sub>C), 2.78 (br, 4H, CH<sub>2</sub>CH<sub>2</sub>), 3.50 (s, 4H, OCH<sub>2</sub>CH<sub>2</sub>O).

Hydrolysis of PEG-PMOSu 6 was conducted by slowly adding 2M NaOH (0.6 ml) dropwise to a stirred solution of the block polymer precursor 6 (0.1 g, 0.54 mmol) in DMF (0.5 ml) in a 10 ml round bottom flask. The reaction mixture was heated to 60 °C for 16 h and more water (5.0 ml) was added. The dilute solution was then dialysed using Visking tubing (MWCO 12,000-14,000) and the dialysate was freeze dried to give PEG-PMAA-Na salt 7 as white solid product PEG<sub>5000</sub>-PMAA-Na salt (75% yield);  $M_n = 29,360 \text{ g}\cdot\text{mol}^{-1}$   $M_w/M_n = 1.28$  (Triple detection-SEC; NaNO<sub>3</sub> 0.2 M/ 10% CH<sub>3</sub>CN).  $^1\text{H}$ -NMR (D<sub>2</sub>O)  $\delta$  0.87-0.97 (br s, 3H, CH<sub>3</sub>) 1.63, 1.89 (br, m, 2H, CH<sub>2</sub>) and 3.58 (s, 4H, OCH<sub>2</sub>CH<sub>2</sub>O) confirming the hindered ester bond to the PEG<sub>5000</sub>-block was not hydrolysed.

**EXAMPLE C) Preparation of amphotericin B – PMAA-Na preparation:**

**Example C1:**

The polymer, PMOSu (40 mg), was dissolved in DMSO (0.4 ml) and the solution added to a vial containing amphotericin B (the “drug”) (50 mg). Under stirring, 1 M aqueous sodium hydroxide (0.44 ml) was added dropwise to the resulting drug mixture. Typically, some precipitate was observed. Additional water (4.7 ml) was added immediately following the addition of sodium hydroxide and the resulting mixture left to stir at room temperature for 1 h. The reaction solution was then diluted to 8.8 ml and dialysed against water (1 L) for 24 h using Visking dialysis membrane (MWCO 7000, Medicell International). During dialysis the water was changed 6 times. The dialysed solution was filtered through a 0.2 µm filter and then freeze-dried to afford 0.9 g of a yellow solid product. FT-IR (ATR) 1676 cm<sup>-1</sup>, 1568 cm<sup>-1</sup>,

1404 cm<sup>-1</sup>, 1070 cm<sup>-1</sup>, 1012 cm<sup>-1</sup>.

The presence of drug and polymer in the preparations was confirmed by <sup>1</sup>H NMR and FT-IR spectroscopy. The weight percent (wt%) of amphotericin B in the

5 preparations was estimated using UV spectrometry at 419.5 nm against a calibration curve made using amphotericin B obtained from Bristol-Myers Squibb Pharmaceuticals Ltd. The wt% in example C1 was estimated to be 44 to 48%.

Prior to their biological evaluation, aqueous solutions of methacrylic acid sodium

10 salt homo-polymers and co-polymers were treated to remove endotoxin using activated carbon or polymixin B columns. Endotoxin levels were determined using the limulus amebocyte lysate (LAL) assay and the compounds used in the experiments described contained <0.06 endotoxin units/ml. This is the European Community standard for water for injection.

15

**D) Cellular toxicity of methacrylic acid sodium salt homo-polymer and co-polymers on primary cells:**

All experiments were performed on primary human cells.

20 **Example D1: Human red blood cells:**

A 2% v/v solution of human red blood cells was prepared in RPMI 1640. A stock solution of PMAA-Na ("drug") was prepared in RPMI 1640. A 1% solution of Triton X-100 was used as a positive reference for 100% cell lysis. Dextran and poly-L-lysine were used as negative and positive controls respectively. An equal volume 25 of the sample and red blood cells were aliquoted into a 96 well microtitre plate and incubated at 37°C. After 1 h and after 24 h, each sample was centrifuged (2,000g, 10 min) and the supernatant added to a 96 well microtitre plate. The absorbance was measured at 490 nm using a spectrophotometer. The degree of lysis was expressed as a percentage of the 100% lysis caused by Triton X-100. No

30 significant toxicity was seen with the PMAA-Na using human red blood cells from 3 donors (A, B and C) up to a concentration of 2,000 µg/ml after 1 h or after 24 hours as shown in Figure 1.

***Example D2: Human whole blood:***

A 2% v/v solution of human whole blood was prepared from donor D in RPMI 1640. A stock solution of PMAA-Na ("drug") was prepared in RPMI 1640. A 1% solution of Triton X-100 was used as a positive reference for 100% cell lysis.

- 5 Dextran and poly-L-lysine were used as negative and positive controls respectively. An equal volume of the sample and whole blood were aliquoted into a 96 well microtitre plate and incubated at 37°C. After 1 h, 6 h and after 24 h, each sample was centrifuged (500g, 10 min) and the supernatant added to a 96 well microtitre plate. The absorbance was measured at 490 nm using a spectrophotometer. The degree of lysis was expressed as a percentage of the 100% lysis caused by Triton X-100. No significant toxicity was seen with the PMAA-Na using human whole blood up to a concentration of 500 µg/ml after 1 h or after 6 h or after 24 hours as shown in Figure 2.

15 ***Example D3***

***a) Monocyte derived macrophages:***

- Monocytes were separated from fresh blood from single donors and cultured in RPMI 1640, 20 mM L-glutamine, penicillin (200 IU/ml), streptomycin (200 µg/ml) and 10 % human serum and plated at a density of  $1 \times 10^6$  cells/ml. The monocytes 20 were allowed to differentiate to monocyte derived macrophages (MDMs) by a further 3 days of adherence. Media containing PMAA-Na was then added to the cells over the concentration range of 0 - 2,000 µg/ml. The cells were incubated for 71 h prior to the addition of thiazolyl blue (MTT, Sigma 5 mg/ml). The viability of the cell culture was expressed as a percentage of the viability of cells grown in the 25 absence of any compound. Dextran and poly(L-lysine) were used as negative and positive controls respectively. PMAA-Na was not toxic to MDMs at the highest concentration of 2,000 µg/ml tested using the MTT assay and the Trypan blue assay as shown in Figure 3a.

30 ***b) Peritoneal tissue macrophages:***

Human peritoneal cells were cultured in RPMI 1640 medium, 20 mM L-glutamine, 10% mixed donor human serum, 200 IU/ml penicillin and 200 µg/ml streptomycin and their density adjusted to  $1 \times 10^6$  cells/ml. Media containing PMAA-Na was then

added to the cells over the concentration range of 0 - 2,000 µg/ml. The cells were incubated for 71 h prior to the addition of MTT. The viability of the cells was expressed as a percentage of the viability of cells grown in the absence of any compound. Dextran and poly(L-lysine) were used as negative and positive controls respectively. PMAA-Na was not toxic to peritoneal macrophages up to 500 µg/ml. Between 500 µg/m and 2,000 µg/ml, a modest amount of toxicity was seen using the MTT assay and the Trypan blue assays as shown in Figure 3b.

#### **E) Anticoagulant activity:**

10 **Example E1:** The anticoagulant activity was determined as a measure of the prothrombin time (PT), kaolin partial thromboplastin time (APTT), thrombin time (TT), fibrinogen and anti-Xa activity. The compounds were solubilised in phosphate-buffered saline solution and were mixed 50 : 50 with plasma / veronal buffer (1 : 1) and tested. The anti-Xa activity was expressed as units of heparin/ml (HEP (Xa) (V/ml)). PMAA-Na prolonged the APTT and the TT but did not affect the PT. The anti-Xa assay was negative. Therefore, the anticoagulant activity of these molecules was not due to "heparin like" activity. see Table 6.

15

Table 6

## 20 Anticoagulant activity of PMAA-Na:

	Dissolved in	Conc (µg/ml)	PT (sec)	APTT (sec)	TT (sec)	Fibrinogen (g/L)	Anti-Xa (V/ml)
Control	Plasma/veronal buffer		16.7	40	14	1.31	0
PMAA-Na	Plasma/veronal buffer	500	14.9	144	67	0.47	0

Peritoneal cells from patients on continuous ambulatory peritoneal dialysis (CAPD) were cultured with each compound and culture supernatants harvested after 36 h of incubation. Pro-inflammatory chemokines and pro-inflammatory cytokines were measured by EIA (R & D Systems).

5

***Example F1:***

Human peritoneal cells from 3 donors (A, B and C) were cultured with PMAA-NA (500 µg/ml) and culture supernatants harvested after 36 h were analysed for MIP-1 $\beta$ . All reagents and the PMAA-Na contained <0.06 endotoxin units/ml (EU/ml) as determined using the *Limulus* amebocyte lysate assay (Pyrotell, Associates of Cape Cod, US). This is the European Community standard value for water for injection. There was a significant release of MIP-1 $\beta$  in the presence of PMAA-Na as shown in Figure 4.

15   ***Example F2:***

Human peritoneal cells from 2 donors (A and B) were cultured with PMAA-NA (500 µg/ml and 2,000 µg/ml) and culture supernatants harvested after 36 h were analysed for TNF- $\alpha$ . All reagents and the PMAA-Na contained <0.06 endotoxin unit/ml. There was a significant release of TNF- $\alpha$  in the presence of PMAA-Na at both concentrations as shown in Figure 5.

***Example F3:***

Human peritoneal cells were cultured with PMAA-NA (500 µg/ml) and culture supernatants harvested after 36 h were analysed for the pro-inflammatory chemokines MIP-1 $\alpha$ , MIP-1 $\beta$  and IL-8, and for the pro-inflammatory cytokines TNF- $\alpha$ , IL-1 $\beta$  and IL-6. All reagents and the PMAA-Na contained <0.06 endotoxin unit/ml. The results with cells from up to 3 different human donors (A, B and C) are shown in Figure 6. The release of these chemokines and cytokines from tissue antigen presenting cells was at a level that would promote a pharmacological Th1 response in man but not high enough to cause significant adverse side-effects in man.

***Example F4:***

Blood-derived monocyte derived macrophages were cultured with PMAA-Na at concentrations of up to 2,000 µg/ml. All reagents and the PMAA-Na contained <0.06 endotoxin unit/ml. No release of MIP-1 $\beta$  was seen. The results with cells from 3 different human donors (A, B and C) are shown in Figure 7. There is  
5 therefore a differential immuno-modulatory effect of PMAA-Na on cells of blood monocyte origin compared to cells of macrophage origin and other antigen presenting cells (e.g., dendritic cells) from tissue body based compartments.

**G) Lack of Th1 adjuvant activity of commercially available PMAA-Na:**

10 **Example G1:**

Human peritoneal macrophages were cultured with commercially available PMAA-Na (Polymer Standards Service, Germany) of a range of MWt's ( $M_n = 1,300$ , 22,100 and 129,000 g/mol) and narrow molecular weight distribution. All reagents and the PMAA-Na contained <0.06 endotoxin unit/ml. One of the commercially  
15 available PMAA-Na possessed a MWt comparable to our synthetic preparation of PMAA-Na ( $M_n = 22,000$  g/mol). The culture supernatants were harvested after 36 h. There was no release of MIP-1 $\beta$ . Figure 8 shows the individual results from 2 human donors (A and B).

20 **Example G2:**

Human peritoneal macrophages were cultured with commercially available PMAA-Na (Polymer Standards Service, Germany) of a range of MWt's ( $M_n = 1,300$ , 22,100 and 129,000 g/mol) and narrow molecular weight distribution. One of the commercially available PMAA-Na possessed a MWt comparable to our synthetic  
25 preparation of PMAA-Na ( $M_n = 22,000$  g/mol). All reagents and the PMAA-Na contained <0.06 endotoxin unit/ml. The culture supernatants were harvested after 36 h. There was no release of TNF- $\alpha$ . Figure 9 shows the individual results from 2 human donors (A and B).

30 **H) Cellular toxicity of the amphotericin B – PMAA-Na preparation on primary human cells:**

**Example H1: Red blood cells:**

The method was as described for Example D1. The comparison was made between clinical grade amphotericin B and the amphotericin B – PMAA-Na preparation (both called "drug" in the Figure). Stock solutions of the compounds for testing were prepared in MGM. Human red cell lysis was determined after 1 hour (Figure 10), 6 hour (Figure 11) and 24 hour (Figure 12) incubation in 3 different donors (A, B and C). No toxicity was seen with the amphotericin B – PMAA-Na preparation after a 1 hour incubation. After a 6 hour incubation, the toxicity of the amphotericin B – PMAA-Na preparation was considerably less than that of clinical grade amphotericin B. When the incubation time was increased to 24 h, the toxicity of the clinical grade amphotericin B increased to 100% but there was no further increase in the toxicity of the amphotericin B – PMAA-Na preparation.

***Example H2: Red blood cells:***

15 The method was as described for Example H1. The degree of red cell lysis by the amphotericin B – PMAA-Na preparation ("drug") was determined after a 1 hour and 24 hour incubation for concentrations up to 1,000 µg/ml. The results are shown in Figure 13.

20 ***Example H3: Peripheral blood mononuclear cells:***

Peripheral blood mononuclear cells (PBMCs) cells were suspended in RPMI medium, 10% mixed donor human serum, 200 IU/ml penicillin and 200 µg/ml streptomycin at  $1 \times 10^5$  cells and cultured in 96-well tissue culture plates at 37°C with 5% CO<sub>2</sub>. Media containing the amphotericin B – PMAA-Na preparation ("drug") was added to the cells over the concentration range of 0 - 70 µg/ml in a final volume of 100 µl/well. The cells were incubated for 1 day or 2 days or 6 days prior to the addition of MTT (5 mg/ml). The MTT solution was removed after 1 h and DMSO (100 µl) added to dissolve the MTT crystals. The optical density was measured at 550 nm using a plate reader (Molecular Devices, Wokingham, UK).

25 The viability of the cell culture was expressed as a percentage of the viability of the cells grown in the absence of any compound. Dextran and poly(L-lysine) were used as negative and positive controls respectively. The compound was not toxic to peripheral blood mononuclear cells at the highest concentration of 70 µg/ml

tested using the MTT assay and the Trypan blue assay after 1 day of culture, 2 days of culture or after 6 days of culture. Figure 14 shows the individual results from up to 3 representative human donors. The results from each donor are shown as line graphs in Figure 14.

5

***Example H4: Monocyte derived macrophages:***

Monocyte derived macrophages (MDM) cells were separated from fresh blood from single donors by density gradient centrifugation and cultured in macrophage growth medium (MGM) containing RPMI 1640, 20 mM L-glutamine, penicillin (250

10 IU/ml), streptomycin (250 µg/ml) and 10 % human serum. They were plated at a density of  $1 \times 10^6$  cells/ml and allowed to differentiate to MDMs for 3 days. Media containing the compounds ("drugs") was then added to the cells up to a concentration of 125 µg/ml. The cells were incubated for 2 days or 3 days prior to the addition of MTT. Cell viability was expressed as a percentage of the viability of cells grown in the absence of any compound. Dextran and poly(L-lysine) were used as negative and positive controls respectively. The amphotericin B – PMAA-Na preparation was less toxic than clinical grade amphotericin B at all of the concentrations up to 125 µg/ml tested using the MTT assay and the Trypan blue assay after both 2 days and after 3 days of culture as shown in Figure 15.

20

***J) Determination of the anti-leishmania activity of the amphotericin B – PMAA-Na preparation against Leishmania promastigotes:***

***Example J1:***

*Leishmania mexicana* promastigotes were used for these experiments. They were maintained in Schneider's Drosophila growth medium (Invitrogen) supplemented with 15% fetal calf serum (heat inactivated at 56°C for 1 hour) and gentamicin (1 mg/100 ml). The parasite concentration was adjusted to  $2 \times 10^6$  parasites/ml.

Doubling dilutions of the test compound were prepared at twice the desired final concentration in growth media. An equal volume of the drug and the parasite

30 suspension were then mixed in a 96 well plate and incubated for 24 h at 26°C. At this time, MTT (5 mg/ml) was added and the plate incubated for a further 24 h at 26°C. The plate was then centrifuged (2000g, 5 min), the supernatant discarded and the pellet resuspended in 100 µl DMSO. Absorbance was measured at 570

nm using a spectrophotometer. The results were expressed as the percentage viability of the promastigotes with 100% viability being determined using the OD of the untreated, replicating promastigotes in the control wells.

- 5 The 50% lethal dose ( $LD_{50}$ ) of clinical grade amphotericin B for *Leishmania mexicana* promastigotes was 0.14 µg/ml (Figure 16 inset). The 90% lethal dose ( $LD_{90}$ ) of clinical grade amphotericin B for *Leishmania mexicana* promastigotes was 1.49 µg/ml (Figure 16 inset). The results from 3 experiments with the amphotericin B – PMAA-Na preparation are shown in Figure 16. The 50% lethal dose ( $LD_{50}$ ) of the amphotericin B – PMAA-Na preparation for *Leishmania mexicana* promastigotes was 0.10 - 0.19 µg/ml (Figure 16). The 90% lethal dose ( $LD_{90}$ ) of amphotericin B – PMAA-Na preparation for *Leishmania mexicana* promastigotes was 1.02 - 1.49 µg/ml (Figure 16). The activity of the amphotericin B – PMAA-Na preparation against *Leishmania mexicana* promastigotes was 10 similar to that of clinical grade amphotericin B on a weight per weight basis.

**Example J2:**

- Leishmania donovania* promastigotes were used for these experiments. They were maintained in Schneider's Growth Media 199 supplemented with 15% fetal calf serum (heat inactivated at 56°C for 1 hour) and gentamicin (1 mg/100 ml). The parasite concentration was adjusted to  $2 \times 10^6$  parasites/ml. Doubling dilutions of the test compound were prepared at twice the desired final concentration in growth media. An equal volume of the drug and the parasite suspension were then mixed in a 96 well plate and incubated for 24 h at 26°C. At this time, MTT (5 mg/ml) was added and the plate incubated for a further 24 h at 26°C. The plate was then centrifuged (2000g, 5 min), the supernatant discarded and the pellet resuspended in 100 µl DMSO. Absorbance was measured at 570 nm using a spectrophotometer. The results were expressed as the percentage viability of the promastigotes with 100% viability being determined using the OD of the untreated, replicating promastigotes in the control wells.

The 50% lethal dose ( $LD_{50}$ ) of clinical grade amphotericin B for *Leishmania donovani* promastigotes was 0.08 µg/ml (Figure 17 inset). The 90% lethal dose

(LD<sub>90</sub>) of clinical grade amphotericin B for *Leishmania donovana* promastigotes was 1.91 µg/ml (Figure 17 inset). The results from 3 experiments with the amphotericin B – PMAA-Na preparation are shown in Figure 17. The 50% lethal dose (LD<sub>50</sub>) of amphotericin B – PMAA-Na preparation for *Leishmania donovani* promastigotes was 0.10 - 0.17 µg/ml (Figure 17). The 90% lethal dose (LD<sub>90</sub>) of amphotericin B – PMAA-Na preparation for *Leishmania donovani* promastigotes was 0.98 - 1.28 µg/ml (Figure 17). The activity of the amphotericin B – PMAA-Na preparation against *Leishmania donovani* promastigotes was therefore similar to that of clinical grade amphotericin B on a weight-per-weight basis.

10

**K) Determination of the anti-leishmania activity of the amphotericin B – PMAA-Na preparation against *Leishmania amastigotes*:**

**Example K1:**

*Leishmania mexicana* amastigotes were used to infect human monocyte derived macrophages that were maintained in RPMI 1640 (Invitrogen) supplemented with 10% human serum (heat inactivated at 56°C for 1 hour) and 200 IU/ml penicillin and 200 µg/ml streptomycin. The cells were plated at 10<sup>6</sup> cells/ml into Lab-Tek Chamber Slides (Nunc) and incubated at 37°C/5% CO<sub>2</sub> for 3 days. The media was then aspirated from the chamber slides and an equal volume of fresh media added back in which the amastigote concentration had been adjusted to give a parasite:cell infection ratio of 5:1. The cells were then incubated at 32°C for 20 h to enable infection of the macrophages to become established. Media was then aspirated from the chamber slides and replaced with doubling dilutions of the test compound. The cells were then incubated at 32°C for 72 h. After washing with PBS, the chambers were detached, the slides allowed to dry and then fixed in methanol. Each slide was then stained with Giemsa and examined microscopically. A total of 250 cells were counted to determine the number of infected and uninfected cells.

30 The infection index was calculated as the average number of parasites/cell multiplied by the percentage of infected cells. A 50% inhibition of intracellular *Leishmania mexicana* amastigote growth was achieved with 0.18 – 0.32 µg/ml of the amphotericin B – PMAA-Na preparation (Figure 18) compared to 0.14 µg/ml of

clinical grade amphotericin B (Figure 19a) or 0.45 µg/ml of Ambisome (Figure 19b). A 90% inhibition of intracellular *Leishmania mexicana* amastigote growth was achieved using 1.18 - 1.55 µg/ml of the amphotericin B – PMAA-Na preparation compared to 0.95 µg/ml of clinical grade amphotericin B or 3.89 µg/ml of Ambisome.

This graphs in Figures 19 and 20 compare the slopes of relative activity of clinical grade amphotericin B and Ambisome (liposomal amphotericin B, Gilead Sciences, Great Abingdon, Cambridge, UK) and amphotericin B – PMAA-Na preparation against intracellular *Leishmania mexicana* amastigotes. They demonstrate that the killing curve for amphotericin B – PMAA-Na preparation is steeper than the killing curve for Ambisome.

**Example K2:**

*Leishmania donovani* amastigotes were used to infect human monocyte derived macrophages that were maintained in RPMI 1640 (Invitrogen) supplemented with 10% human serum (heat inactivated at 56°C for 1 hour) and 200 IU/ml penicillin and 200 µg/ml streptomycin. The cells were plated at  $10^6$  cells/ml into Lab-Tek Chamber Slides (Nunc) and incubated at 37°C/5% CO<sub>2</sub> for 3 days. The media was then aspirated from the chamber slides and an equal volume of fresh media added back in which the amastigote concentration had been adjusted to give a parasite:cell infection ratio of 5:1. The cells were then incubated at 32°C for 20 h to enable infection of the macrophages to become established. Media was then aspirated from the chamber slides and replaced with doubling dilutions of the test compound. The cells were then incubated at 32°C for 72 h. After washing with PBS, the chambers were detached, the slides allowed to dry and then fixed in methanol. Each slide was then stained with Giemsa and examined microscopically. A total of 250 cells were counted to determine the number of infected and uninfected cells.

The infection index was calculated as the average number of parasites/cell multiplied by the percentage of infected cells. A 50% inhibition of intracellular *Leishmania donovani* amastigote growth was achieved with 0.30 – 0.71 µg/ml of

the amphotericin B – PMAA-Na preparation (Figure 21) compared to 0.54 µg/ml of clinical grade amphotericin B (Figure 22a) or 1.96 µg/ml of Ambisome (Figure 22b). A 90% inhibition of intracellular *Leishmania donovani* amastigote growth was achieved using 2.18 – 3.18 µg/ml of the amphotericin B – PMAA-Na preparation  
5 compared to 2.31 µg/ml of clinical grade amphotericin B or >8 µg/ml of Ambisome.

**L) The interferon- $\gamma$  releasing, Th1 adjuvant activity of the amphotericin B - PMAA-Na preparation:**

**Example L1:**

10 Human peritoneal cells that included macrophages and dendritic cells were cultured in macrophage growth medium with each compound. All reagents and compounds contained <0.06 endotoxin unit/ml. The culture supernatants were harvested 24 h later. Interferon- $\gamma$  was measured by EIA. The release of the Th1 promoting cytokine, interferon- $\gamma$ , from peritoneal macrophages was significantly higher in the presence  
15 of the amphotericin B – PMAA-Na preparation (100 µg/ml) compared to cells only control, clinical grade amphotericin B, commercial PMAA-Na, or the PMAA-Na as shown in Figure 23. The level of interferon- $\gamma$  release with the endotoxin free amphotericin B – PMAA-Na preparation would be sufficient to promote a pharmacological Th1 response in man but would not be high enough to cause  
20 significant adverse side-effects in man.

**Example L2:**

The purpose of the experiments described in this section was to establish whether a preparation of an antigen and PMAA-Na could also elicit a Th1 helper response.

25 The tuberculin-PMAA-Na preparation was made as follows. The homo-polymer, PMAA-Na (30 mg), prepared as described in **Examples A1 to A4** was added to tuberculin purified protein derivative BP (10 ml, 100,000 units/ml, Evans Vaccines) and the resulting solution was stirred for 1.5 h at room temperature. The solution  
30 was then dialysed against 1 litre of water using Visking dialysis membrane (MWCO 7000, Medicell International) over 24 h, changing the water six times. The dialysed solution was filtered through a 0.2 µm filter and freeze-dried to afford an off-white

solid product (20 mg). FT-IR (ATR) 1648 cm<sup>-1</sup>, 1545 cm<sup>-1</sup>, 1450 cm<sup>-1</sup>, 1398 cm<sup>-1</sup>, 1259 cm<sup>-1</sup>.

PBMCs were then isolated from whole blood using Ficoll-Paque and resuspended in RPMI 1640 supplemented with 10% human serum, 200 µg/ml penicillin and 200 IU/ml streptomycin. All reagents and compounds contained <0.06 endotoxin unit/ml as determined using the *Limulus* amebocyte lysate assay (Pyrotell, Associates of Cape Cod, US). The cells (10<sup>5</sup> cells/well) and each compound were mixed in duplicate and incubated at 37°C / 5% CO<sub>2</sub> for 4 days. [<sup>3</sup>H]-Thymidine (specific activity 20-30 Ci/mmol; Amersham Biosciences, UK) was added at 1 µCi/well for a further 18 hours. The cells were then harvested and proliferation determined using a liquid scintillation counter. The results were expressed as the mean counts/minute (cpm) ± sem. Figure 24a shows that there was significantly increased proliferation of T lymphocytes when they were cultured with 1 µg/ml of the tuberculin-PMAA-Na preparation. There was even more proliferation of T lymphocytes when they were cultured with 10 µg/ml of tuberculin-PMAA-Na preparation ( $P = 0.001$ ).

Figure 24b shows the results for donor B. There was significantly more T lymphocyte proliferation with 10 µg/ml of the tuberculin-PMAA-Na preparation than with 10 µg/ml of tuberculin antigen ( $P = 0.002$ ). This shows that the tuberculin-PMAA-Na preparation causes significantly more T lymphocyte proliferation than the tuberculin antigen on its own.

**Example L3: ELISpot assay for Interferon-γ secreting cells:**

Human PBMCs were isolated and adjusted to 2 × 10<sup>5</sup> cells/well in RPMI 1640 supplemented with 10% human serum, 200 µg/ml penicillin and 200 IU/ml streptomycin. All reagents and the compounds contained <0.06 endotoxin unit/ml. The PBMCs and the compounds were mixed in the wells of an ELISpot PVDF-backed microplate coated with the human interferon-γ monoclonal antibody (R&D Systems, UK). Unstimulated cells were used as the negative control and recombinant human interferon-γ was used in the positive control. The plate was incubated for 24 hours at 37°C / 5% CO<sub>2</sub>. The manufacturer's instructions were

then followed to develop the microplate and the number of positive spots for interferon- $\gamma$  counted. Each spot represented a single interferon- $\gamma$  secreting cell. Figure 25 shows that the tuberculin PPD – PMAA-Na preparation was significantly more effective than tuberculin antigen alone in stimulating interferon- $\gamma$  release from primary human T lymphocytes. This increase in interferon- $\gamma$  secretion from T lymphocytes was seen with 50  $\mu$ g/ml of the tuberculin PPD – PMAA-Na preparation and with 100  $\mu$ g/ml of the tuberculin PPD – PMAA-Na preparation.

**M) Determination of the anti-leishmania activity of the amphotericin B – PMAA-Na preparation against Leishmania donovani in a mouse model of visceral leishmaniasis:**

**Example M1:**

*Leishmania donovani* strain (MHOM/ET/67/L82) amastigotes were collected from the spleens of heavily infected donor Syrian Hamsters-*Mesocritetus auratus*. An inoculum containing  $7.5 - 10 \times 10^7$  amastigotes/mL was prepared. Female BALB/c mice (20 g) that were specified pathogen free were infected intravenously with 200  $\mu$ L (equivalent to  $1.5 - 2 \times 10^7$  amastigotes) on day 0. The end point of infection was evaluated by microscopic reading on day 14 to check for the level of infection in one mouse, and on day 21 post-infection of Giemsa stained liver impressions to determine the total parasite burden.

The following compounds were administered intravenously according to the following regimens:-

- a) untreated control after infection.
- 25 b) 0.5 mg/kg of blank liposome on days 14, 16 and 18 after infection.
- c) 8 mg/kg of PMAA-Na on days 14, 16 and 18 after infection.
- d) 1 mg/kg of clinical grade amphotericin B on days 14, 16 and 18 after infection.
- e) 0.5 mg/kg of AmBisome on days 14, 16 and 18 after infection.
- 30 f) 0.5 mg/kg of amphotericin B – PMAA-Na preparation on days 14, 16 & 18 after infection.
- g) 1 mg/kg of amphotericin B – PMAA-Na preparation on days 14, 16 and 18 after infection.

h) 2 mg/kg of amphotericin B – PMAA-Na preparation on days 14, 16 and 18 after infection.

There were 5 animals in each group. The carrier solution for the intravenous 5 injections of PMAA-Na and for amphotericin B – PMAA-Na preparation was 5% dextrose. All drug preparations were filtered using a 0.2 µL syringe filter. The intravenous volume of injection was 200 µL per injection.

Groups of mice were weighed before and after treatment as a measure of toxicity 10 and the percentage weight change noted. On day 21 after infection (which was 3 days after the completion of treatment), the parasite burden was determined microscopically from methanol fixed liver impression slides stained with 10% Giemsa. The number of amastigotes/500 liver cells were counted microscopically and the results expressed as a percentage of the untreated control.

15 Figure 26 shows that the blank liposomes and the PMAA-Na had no anti-leishmanial activity. There was significant killing activity ( $P<0.0001$ ) by clinical grade amphotericin B, Ambisome, amphotericin B – PMAA-Na preparation at 1 mg/kg, and amphotericin B – PMAA-Na preparation at 2 mg/kg of *Leishmania* 20 *donovani* amastigotes compared to the controls. The anti-leishmanial activities of the clinical grade amphotericin B, Ambisome, and the amphotericin B – PMAA-Na (2 mg/kg) were not significantly different from each other ( $P<0.05$ ). These results therefore show that the amphotericin B – PMAA-Na preparation was as effective as Ambisome in this animal model of visceral leishmaniasis.

**CLAIMS:**

1. A complex that comprises a narrow molecular weight distribution polymer that includes units derived from an acrylic acid or a salt thereof, and
  - (i) a substance that has pharmacological activity against a pathogenic organism,  
5 or
    - (ii) a substance that has pharmacological activity against a cancer, or
    - (iii) one or more agents selected from antigens and immunogens.
- 10 2. A complex as claimed in claim 1, wherein the pathogenic organism is predominantly but not exclusively an intracellular organism.
- 15 3. A complex as claimed in claim 2, wherein the pathogenic organism is an intracellular organism that exists and/or persists in cells of macrophage origin and/or in other antigen presenting cells such as dendritic cells.
4. A complex as claimed in claim 1, wherein the pathogenic organism is selected from the following organisms :
  - a) Organisms that cause superficial mycoses, including ringworm; tinea; thrush;  
20 Malassezia infections, including pityriasis versicolor, Malassezia folliculitis, seborrhoeic dermatitis and Scytalidium infections; Otomycosis; and Keratomycosis.
  - b) Candida species that cause invasive and chronic fungal infections, including  
25 Candida albicans, Candida tropicalis and Candida glabrata; Aspergillus species, including Aspergillus fumigatus, Aspergillus flavus and Aspergillus niger; Cryptococcus neoformans; Mucormycosis, for example, caused by species of Absidia, Rhizopus and Rhizomucor; Fusarium species; Trichosporon species; Blastomycosis; Sporothrix species; Sporotrichum species; Histoplasmosis, for  
30 example, caused by Histoplasma capsulatum var. capsulatum; African histoplasmosis, for example, caused by Histoplasma capsulatum var. duboisii; Blastomycosis, for example, caused by Blastomyces dermatitidis; Coccidioidomycosis, for example, caused by Coccidioides immitis;

Paracoccidioidomycosis, for example, caused by *Paracoccidioides brasiliensis*; and Infections caused by *Penicillium marneffei*.

- c) Organisms that cause mycobacterial diseases, for example, tuberculosis and
  - 5 leprosy caused by members of the mycobacterial family, for example, *Mycobacterium tuberculosis*, atypical mycobacteria, and *Mycobacterium leprae*.
  - d) Members of the schistosoma family that cause Schistosomiasis, for example, *Schistosoma haematobium*, *Schistosoma mansoni*, *Schistosoma japonicum*,
  - 10 *Schistosoma intercalatum*, and *Schistosoma mekongi*.
  - e) Organisms that cause typhoid and paratyphoid fevers, for example, members of the salmonella family of serotypes A, B, C and D.
  - 15 f) Organisms that cause toxoplasmosis, for example, *Toxoplasma gondii*.
  - g) Organisms that cause Human African Trypanosomiasis, for example, *Trypanosoma brucei gambiense* or *Trypanosoma brucei gambiense*.
  - 20 h) Organisms that cause American Trypanosomiasis, for example, *Trypanosoma cruzi*.
  - i) Organisms that cause malaria, for example, *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale* and *Plasmodium malariae*.
  - 25 j) Organisms that cause HIV and HTLV infections.
  - k) Organisms that cause *Pneumocystis carinii* infections.
- 30 5. A complex as claimed in claim 1, wherein the pathogenic organism causes leishmaniasis.

6. A complex as claimed in claim 1, wherein the substance that has pharmacological activity against the pathogenic organism is capable of killing or disrupting an organism as defined in any one of claims 2 to 5.
- 5 7. A complex as claimed in claim 6, wherein the pharmacologically active substance is amphotericin B.
8. A complex as claimed in claim 1, wherein an antigen or immunogen is derived directly or indirectly from an organism that causes tuberculosis, tetanus, 10 anthrax, cholera, diphtheria, measles, mumps, rubella, Hepatitis A, Hepatitis B, influenza, herpes zoster, poliomyelitis, rabies, smallpox, yellow fever, varicella, herpes zoster, herpes simplex, influenza or leishmaniasis.
9. A complex as claimed in claim 1, wherein an antigen or immunogen is 15 derived directly or indirectly from Haemophilus influenzae type B, Neisseria meningitidis, Bordetella pertussis, Streptococcus pneumonia, or Salmonella typhi.
10. A complex as claimed in claim 1, wherein an antigen or immunogen is derived directly or indirectly from an organism as defined in any one of claims 2 to 20 6.
11. A complex as claimed in claim 1, claim 9 or claim 10, wherein an antigen or immunogen is obtained from a natural source, or has been produced by recombinant DNA technology or by chemical synthesis, or by any one or more of 25 said methods.
12. A complex as claimed in claim 1, wherein the substance that has pharmacological activity against a cancer is a cytotoxic agent.
- 30 13. A pharmaceutical preparation which comprises a complex as claimed in any one of claims 1 to 12 in admixture or conjunction with a pharmaceutically suitable carrier.

14. A pharmaceutical preparation as claimed in claim 13, which comprises a delivery system adjuvant.
15. A complex as claimed in any one of claims 1 to 14 for use as a medicament.
16. A complex for use in treatment of an infection by a pathogenic organism and/or for inducing an immune response to the pathogenic organism, which complex comprises a narrow molecular weight distribution polymer that includes units derived from an acrylic acid or a salt thereof and a substance that has pharmacological activity against the pathogenic organism.
17. A method of treating an infection by a pathogenic organism in a subject in need of such treatment, which comprises administering to the subject a therapeutically effective amount of a complex comprising a narrow molecular weight distribution polymer that includes units derived from an acrylic acid or a salt thereof and a substance that has pharmacological activity against the pathogenic organism.
18. A method of inducing an immune response to the pathogenic organism in a subject in need thereof, which comprises administering to the subject an effective amount of a complex comprising a narrow molecular weight distribution polymer that includes units derived from an acrylic acid or a salt thereof and a substance that has pharmacological activity against the pathogenic organism.
19. A complex or a method as claimed in any one of claims 16 to 18, wherein the pathogenic organism is as defined in any one of claims 2 to 5.
20. A complex or a method as claimed in any one of claims 16 to 18, wherein the substance that has pharmacological activity against the pathogenic organism is as defined in claim 6.

21. A complex or a method as claimed in any one of claims 16 to 18, for treatment of leishmaniasis and/or for inducing an immune response to an organism that causes leishmaniasis in any of its clinical forms.
- 5 22. A complex or a method as claimed in claim 21, wherein the substance that has pharmacological activity against an organism that causes leishmaniasis is amphotericin B.
- 10 23. A complex or a method as claimed in any one of claims 16 to 22, wherein the immune response comprises therapeutic and/or prophylactic vaccination against the pathogenic organism.
- 15 24. A complex for use in treatment of a cancer, which complex comprises a narrow molecular weight distribution polymer that includes units derived from an acrylic acid or a salt thereof and a substance that has pharmacological activity against the cancer.
- 20 25. A method of treating a cancer in a subject in need of such treatment, which comprises administering to the subject a therapeutically effective amount of a complex comprising a narrow molecular weight distribution polymer that includes units derived from an acrylic acid or a salt thereof and a substance that has pharmacological activity against the cancer.
- 25 26. A method of inducing an immune response to a cancer in a subject in need thereof, which comprises administering to the subject an effective amount of a complex comprising a narrow molecular weight distribution polymer that includes units derived from an acrylic acid or a salt thereof and a substance that has pharmacological activity against the cancer.
- 30 27. A complex or a method as claimed in any one of claims 24 to 26, wherein the substance that has pharmacological activity against the cancer is a cytotoxic agent.

28. A complex or a method as claimed in any one of claims 24 to 27, wherein the immune response comprises therapeutic and/or prophylactic vaccination against the cancer.
- 5 29. A complex that comprises a polymer including units derived from an acrylic acid or a salt thereof and one or more agents selected from antigens and immunogens, for inducing an immune response to the antigen or immunogen.
- 10 30. A method of inducing an immune response to an antigen or immunogen in a subject, which comprises administering to the subject an effective amount of a complex comprising a narrow molecular weight distribution polymer that includes units derived from an acrylic acid or a salt thereof and the antigens or immunogen.
- 15 31. A complex or a method as claimed in any one of claims 10 to 12, wherein the antigen or immunogen is derived directly or indirectly from an organism against which a protective immune response is required.
- 20 32. A complex or a method as claimed in claim 29 or claim 30, wherein the antigen or immunogen is as defined in any one of claims 8 to 11.
- 25 33. A complex or a method as claimed in any one of claims 29 to 32, wherein the immune response comprises therapeutic and/or prophylactic vaccination against the pathogenic organism.
- 30 34. A narrow molecular weight distribution polymer that includes units derived from an acrylic acid or a salt thereof for use with a substance that has pharmacological activity against a pathogenic organism for the treatment of an infection by the pathogenic organism and/or for inducing an immune response to the pathogenic organism.
35. A method of treating an infection by a pathogenic organism in a subject in need of such treatment, which comprises administering to the subject therapeutically effective amounts of a narrow molecular weight distribution

- polymer that includes units derived from an acrylic acid or a salt thereof and a substance that has pharmacological activity against the pathogenic organism.
36. A method of inducing an immune response to the pathogenic organism in a subject in need thereof, which comprises administering to the subject effective amounts of a narrow molecular weight distribution polymer that includes units derived from an acrylic acid or a salt thereof and a substance that has pharmacological activity against the pathogenic organism.
- 10 37. A polymer as claimed in claim 34 or a method as claimed in claim 36, wherein the immune response comprises therapeutic and/or prophylactic vaccination against the pathogenic organism.
- 15 38.. A polymer or a method as claimed in any one of claims 34 to 37, wherein the pathogenic organism is as defined in any one of claims 2 to 5.
39. A polymer or a method as claimed in any one of claims 34 to 38, wherein the substance that has pharmacological activity against the pathogenic organism is as defined in claim 6.
- 20 40. A polymer or a method as claimed in any one of claims 34 to 37 for treatment of leishmaniasis and/or for inducing an immune response to an organism that causes leishmaniasis in any of its clinical forms.
- 25 41. A polymer or a method as claimed in claim 40, wherein the substance that has pharmacological activity against the organism that causes leishmaniasis is amphotericin B.
42. A narrow molecular weight distribution polymer that includes units derived from an acrylic acid or a salt thereof for use with one or more agents selected from antigens and immunogens for inducing an immune response to the antigen or immunogen.

43. A method of inducing an immune response to an antigen or immunogen in a subject in need of such treatment, which comprises administering to the subject therapeutically effective amounts of a narrow molecular weight distribution polymer that includes units derived from an acrylic acid or a salt thereof and the antigen or immunogen.
- 5
44. A polymer or a method as claimed in claim 42 or claim 43, wherein the antigen or immunogen is as defined in claim 31 or claim 32.
- 10 45. A polymer or a method as claimed in any one of claims 42 to 44, wherein the immune response comprises therapeutic and/or prophylactic vaccination.
46. A narrow molecular weight distribution polymer that includes units derived from an acrylic acid or a salt thereof for use with a substance that has pharmacological activity against a cancer for the treatment of the cancer and/or for inducing an immune response to the cancer.
- 15
47. A method of treating a cancer in a subject in need of such treatment, which comprises administering to the subject therapeutically effective amounts of a narrow molecular weight distribution polymer that includes units derived from an acrylic acid or a salt thereof and a substance that has pharmacological activity against the cancer.
- 20
48. A method of inducing an immune response to a cancer in a subject in need thereof, which comprises administering to the subject effective amounts of a narrow molecular weight distribution polymer that includes units derived from an acrylic acid or a salt thereof and a substance that has pharmacological activity against the cancer.
- 25
- 30 49. A polymer as claimed in claim 46 or claim 48, wherein the immune response comprises therapeutic and/or prophylactic vaccination.

50. A polymer or a method as claimed in any one of claims 34 to 49, wherein  
the polymer and the other substance are administered together or separately.
51. A polymer or a method as claimed in claim 50, wherein, when the polymer  
and the pharmacologically active substance are administered separately, they are  
administered substantially simultaneously or one is administered before the other.
52. A narrow molecular weight distribution polymer that includes units derived  
from an acrylic acid or a salt thereof for use as an immune potentiating adjuvant in  
the manufacture of a vaccine.
53. A narrow molecular weight distribution polymer that includes units derived  
from an acrylic acid or a salt thereof for use as an immune potentiating adjuvant in  
the manufacture of a vaccine that comprises an antigen or immunogen against  
which an immune response is to be induced.
54. In a method for the manufacture of a vaccine, the improvement comprising  
the use of a narrow molecular weight distribution polymer that includes units  
derived from an acrylic acid or a salt thereof as an immune potentiating adjuvant.
55. A complex, pharmaceutical preparation, method, or polymer as claimed in  
any one of the preceding claims, wherein the narrow molecular weight distribution  
polymer that includes units derived from an acrylic acid or a salt thereof has a  
polydispersity of 1.7 or less.
56. A complex, pharmaceutical preparation, method, or polymer as claimed in  
claim 55, wherein the polymer has a polydispersity of less than 1.4, for example,  
less than 1.2.
57. A complex, pharmaceutical preparation, method, or polymer as claimed in  
any one of the preceding claims, wherein the polymer has a molecular weight of  
100,000 or less, for example, in the range from 50,000 to 4,000, for example, from  
25,000 to 40,000.

58. A complex, pharmaceutical preparation, method, or polymer as claimed in any one of the preceding claims, wherein the polymer is a poly(methacrylic acid) or a salt thereof.

5

59. A complex, pharmaceutical preparation, method, or polymer as claimed in any one of the preceding claims, wherein the polymer is poly(methacrylic acid) or a salt thereof produced by a process that involves hydrolysis of the precursor poly(*N*-methacryloxy succinimide) (PMOSu) with sodium hydroxide.

10

60. A complex, pharmaceutical preparation, method, or polymer as claimed in claim 59, wherein the poly(*N*-methacryloxy succinimide) (PMOSu) is produced by homogeneous polymerisation of methacryloxy succinimide using a copper mediated atom transfer radical polymerisation method.

15

61. A complex, pharmaceutical preparation, method, or polymer as claimed in any one of claims 1 to 57, wherein the polymer is or comprises units (I) or (II) as defined herein.

20

62. A complex, pharmaceutical preparation, method, or polymer as claimed in any one of claims 1 to 57, wherein the polymer is or comprises units (III) or (IV) as defined herein.

25

63. A complex, pharmaceutical preparation, method, or polymer as claimed in any one of claims 1 to 62, wherein the polymer is produced by homogeneous polymerisation of a succinimide precursor using a copper mediated atom transfer radical polymerisation method.

30

65. A complex as defined in or referred to in any preceding claim, which complex is produced by a method comprising hydrolysing a polymer precursors to form the polymer, wherein the hydrolysis is carried out in the presence of the component (i), (ii) or (iii) as defined in any one of the preceding claims .

66. A complex as claimed in claim 1, which comprises a substance that has pharmacological activity against leishmaniasis and a narrow molecular weight distribution polymer that includes units derived from an acrylic acid or a salt thereof.
- 5
67. A complex as claimed in claim 66, wherein the pharmacologically active substance is amphotericin B.
- 10
68. A complex as claimed in claim 66 or claim 67, wherein the polymer is as defined in any one of claims 55 to 65.
- 15
69. A pharmaceutical preparation that comprises a complex as claimed in any one of claims 66 to 68 in admixture with a pharmaceutically suitable carrier and optionally a delivery system adjuvant.
70. A complex as claimed in any one of claims 66 to 68 or a pharmaceutical preparation as claimed in claim 69, for use in the treatment of leishmaniasis in any of its clinical forms.
- 20
71. A method of treating a leishmaniasis in a subject in need of such treatment, which comprises administering to the subject a therapeutically effective amount of a complex as claimed in any one of claims 66 to 68 or a pharmaceutical preparation as claimed in claim 69.

**COMPLEXES HAVING ADJUVANT PROPERTIES**

5

**ABSTRACT**

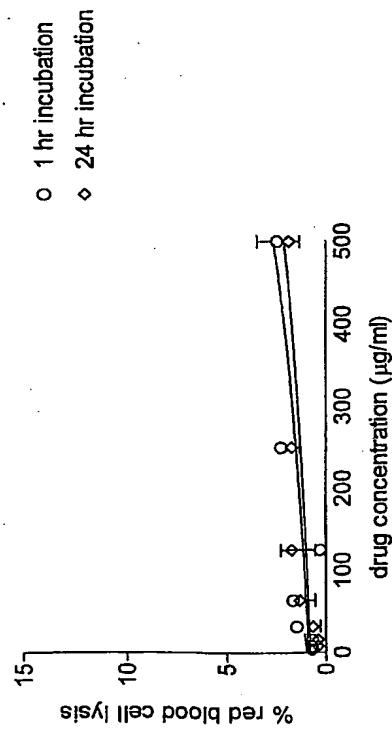
10

- A complex that comprises a narrow molecular weight distribution polymer that includes units derived from an acrylic acid or a salt thereof, and
- (i) a substance that has pharmacological activity against a pathogenic organism, or
  - (ii) a substance that has pharmacological activity against a cancer, or
  - (iii) one or more agents selected from antigens and immunogens
- is useful in treating and/or inducing immunity to the pathogenic organism or the cancer, and for inducing immunity to the antigen or immunogen.

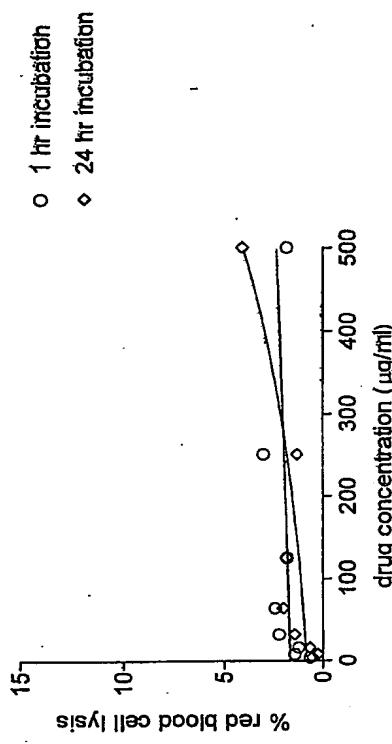
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**Figure 1:**

**Red blood cell lysis in donor A after incubation with PMAA-Na in RPMI**

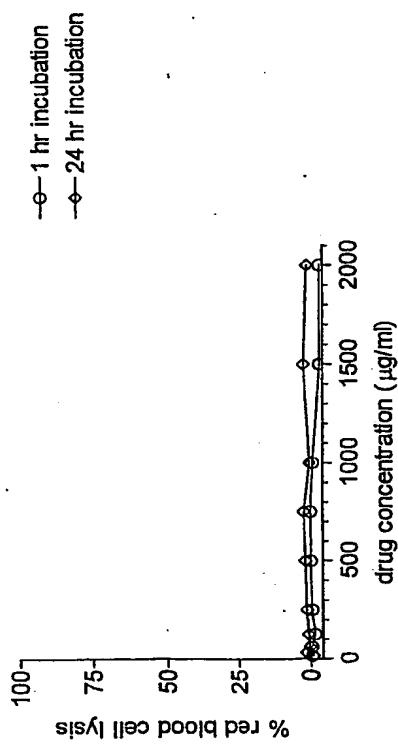


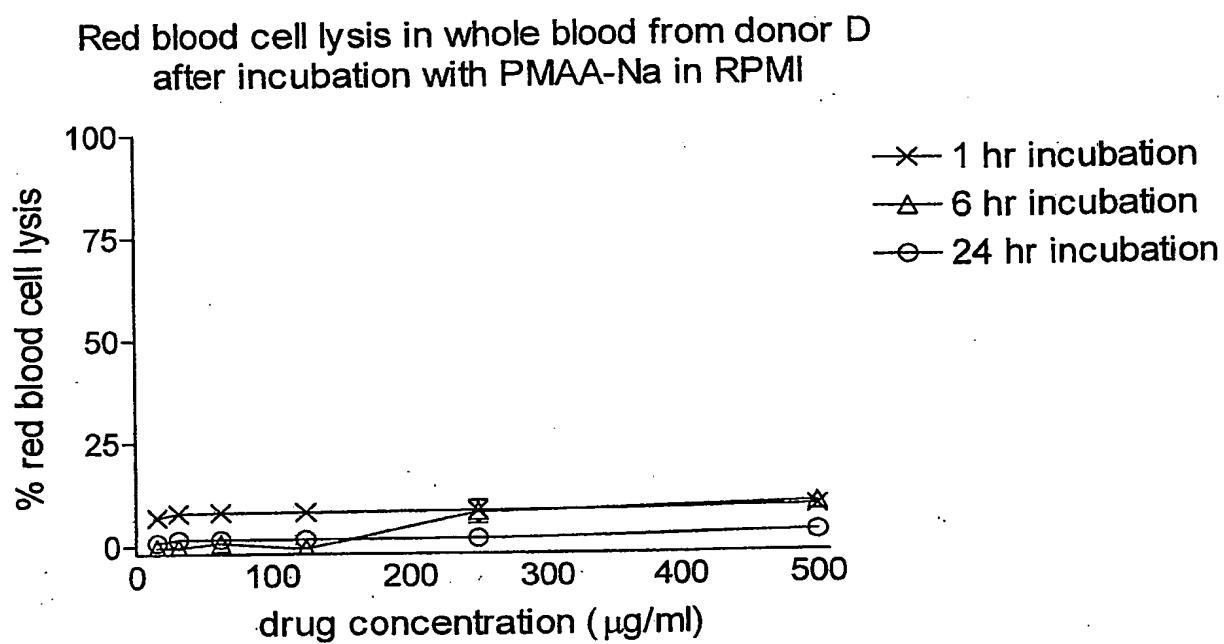
**Red blood cell lysis in donor B after incubation with PMAA-Na in RPMI**



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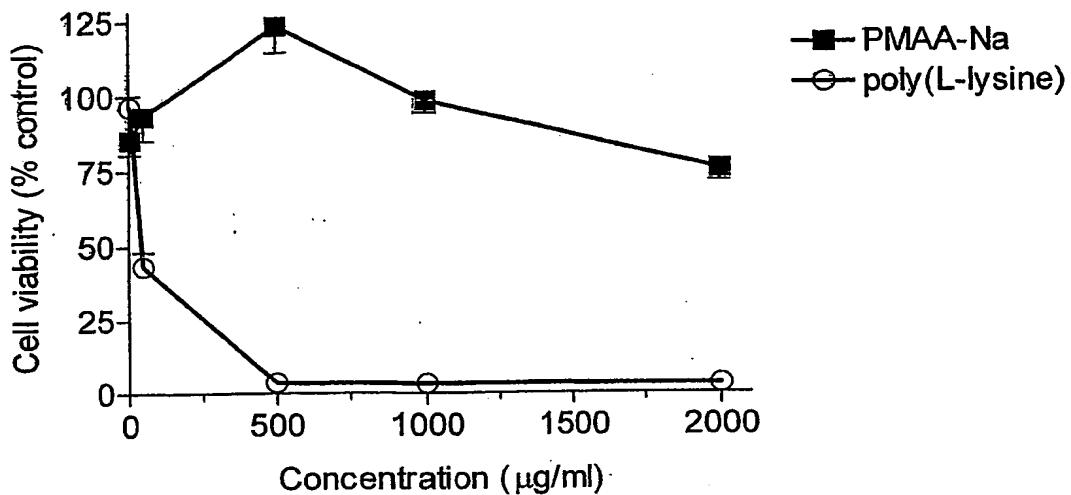
**Red blood cell lysis in donor C after Incubation with PMAA-Na in RPMI**



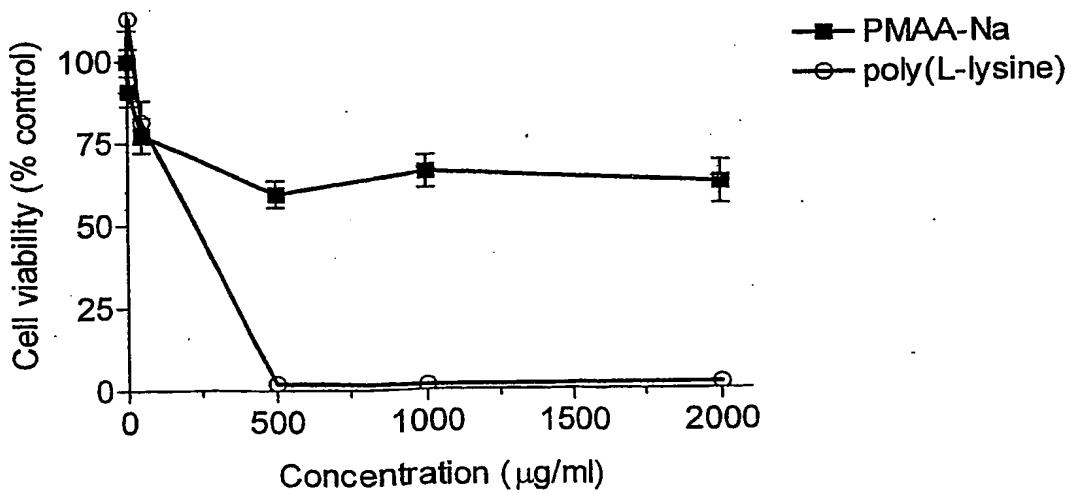
**Figure 2:**

**Figure 3a:**

Lack of toxicity of PMAA-Na on primary human monocyte-derived-macrophages

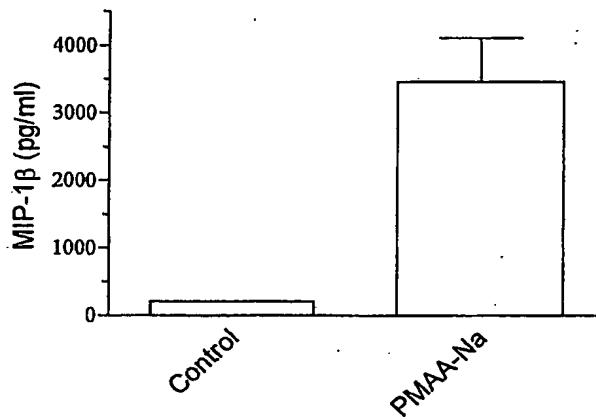
**Figure 3b:**

Lack of toxicity of PMAA-Na on primary human peritoneal macrophages

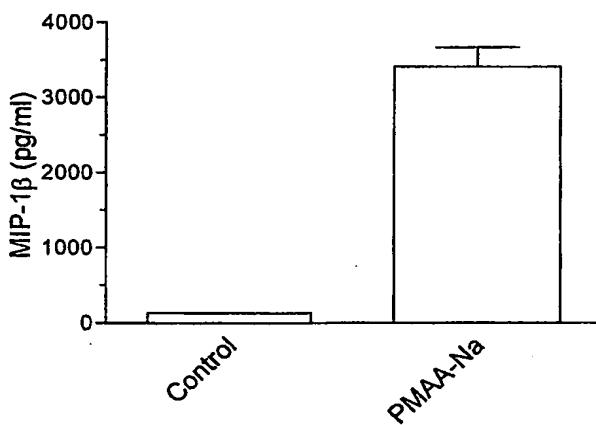


**Figure 4**

**Release of MIP-1 $\beta$  from human peritoneal macrophages by endotoxin free PMAA-Na (500  $\mu$ g/ml) after 36 h from a single donor A**



**Release of MIP-1 $\beta$  from human peritoneal macrophages by endotoxin free PMAA-Na (500  $\mu$ g/ml) after 36h from a single donor B**



**Release of MIP-1 $\beta$  from human peritoneal macrophages by endotoxin free PMAA-Na (500  $\mu$ g/ml) after 36 h from a single donor C**

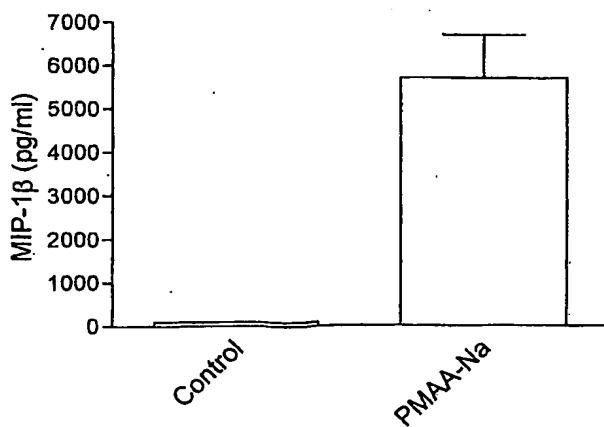
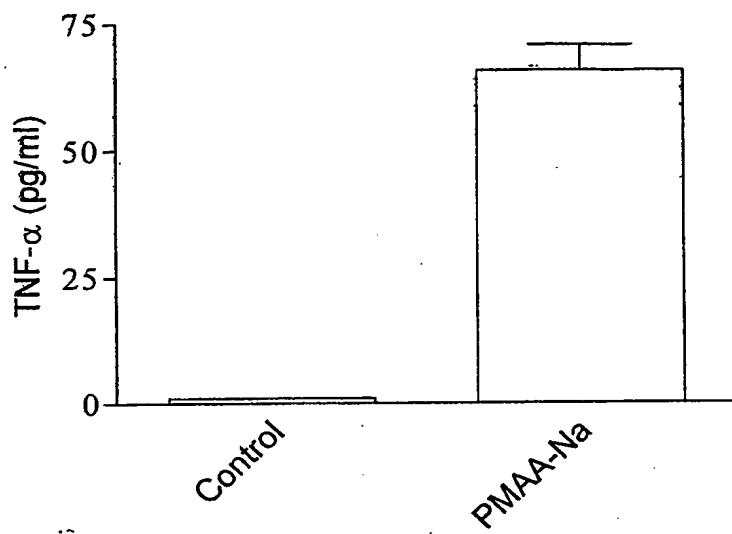
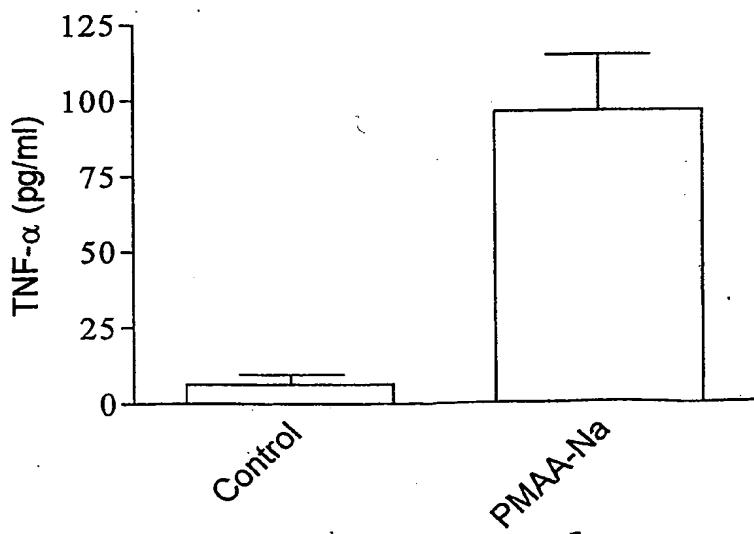


Figure 5:

Release of TNF- $\alpha$  from human peritoneal macrophages by endotoxin free PMAA-Na (2,000  $\mu$ g/ml) after 36 h from a single donor A

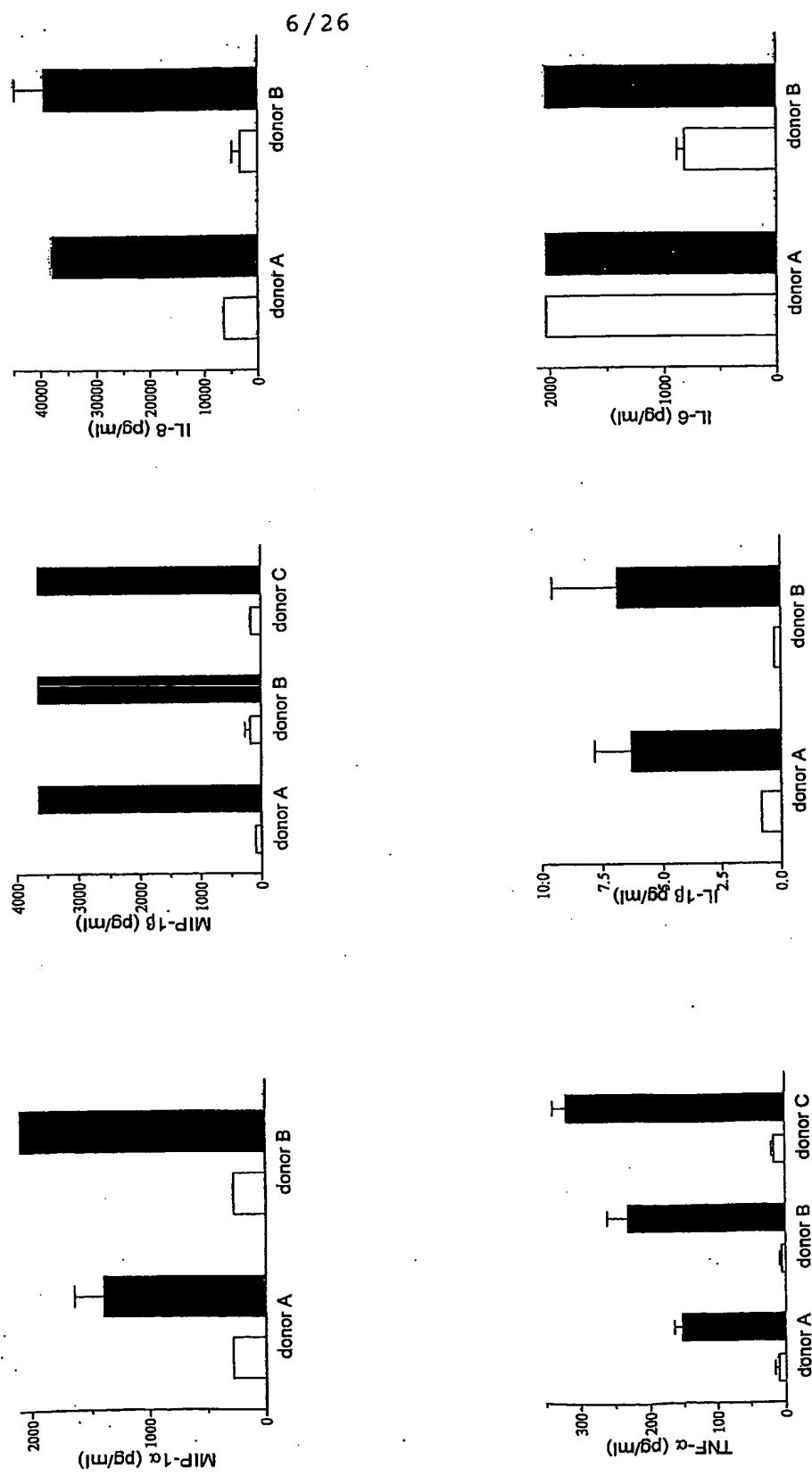


Release of TNF- $\alpha$  from human peritoneal macrophages by endotoxin free PMAA-Na (500  $\mu$ g/ml) after 36 h from a single donor B



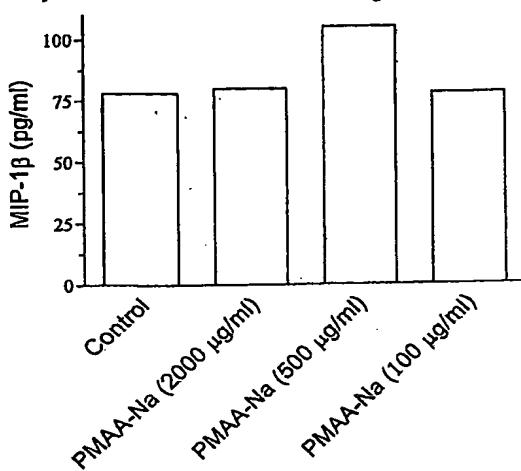
**Figure 6:**

Release of chemokines and cytokines from single donor human peritoneal macrophages  
incubated with media control (□) or with PMAA-Na at 500 µg/ml (■) for 36 h

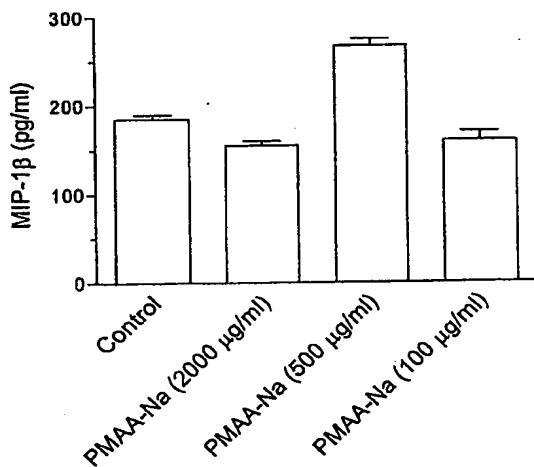


**Figure 7:**

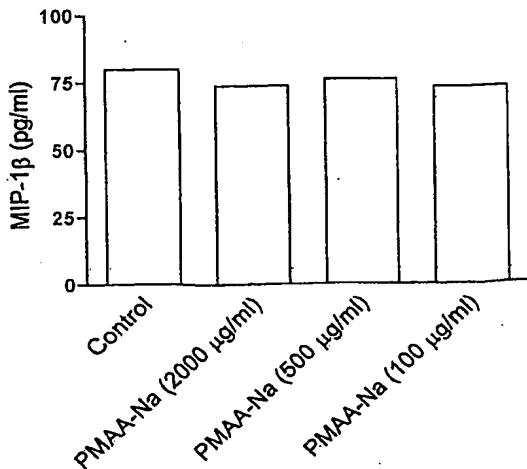
Release of MIP-1 $\beta$  from human monocyte derived macrophages by endotoxin free PMAA-Na after 36 h from a single donor A



Release of MIP-1 $\beta$  from human monocyte derived macrophages by endotoxin free PMAA-Na after 36h from a single donor B

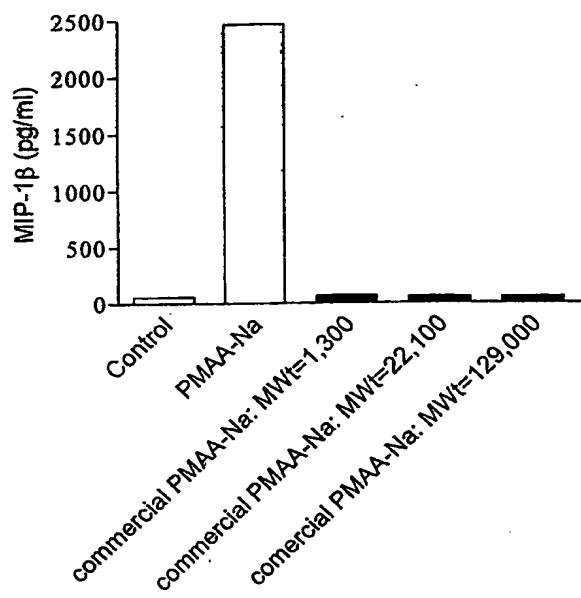


Release of MIP-1 $\beta$  from human monocyte derived macrophages by endotoxin free PMAA-Na after 36 h from a single donor C

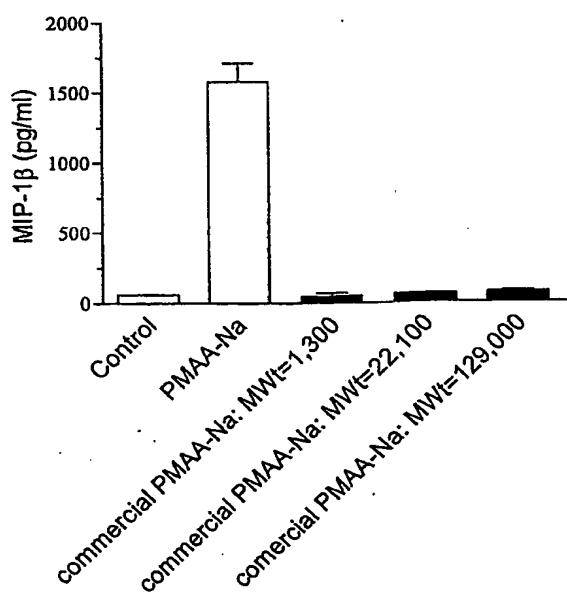


**Figure 8:**

Release of MIP-1 $\beta$  from human peritoneal macrophages by PMAA-Na (500  $\mu\text{g/ml}$ ) but not by commercially available PMAA-Na (500  $\mu\text{g/ml}$ ) after 36 h from donor A

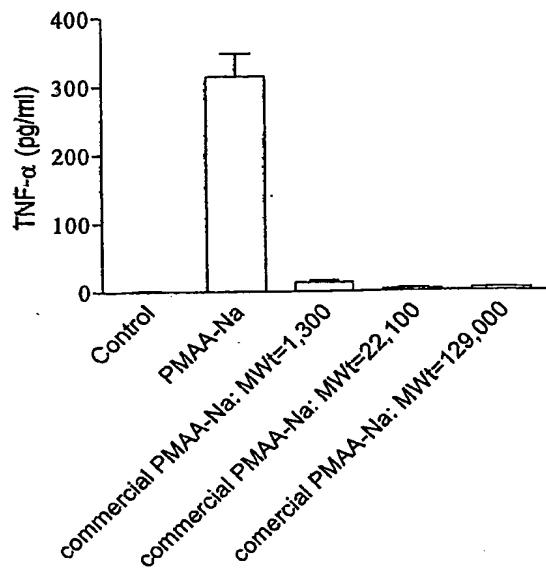


Release of MIP-1 $\beta$  from human peritoneal macrophages by PMAA-Na (500  $\mu\text{g/ml}$ ) but not by commercially available PMAA-Na (500  $\mu\text{g/ml}$ ) after 36 h from donor B

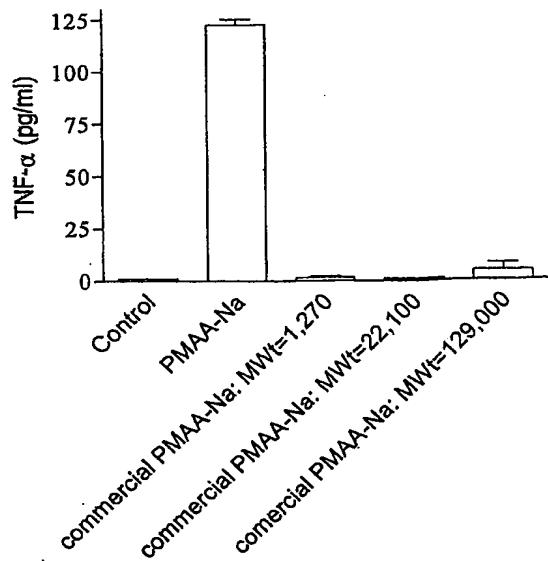


gure 9:

Release of TNF- $\alpha$  from human peritoneal macrophages by PMAA-Na (500  $\mu\text{g/ml}$ ) but not by commercially available PMAA-Na (500  $\mu\text{g/ml}$ ) after 36 h with cells from a single donor A

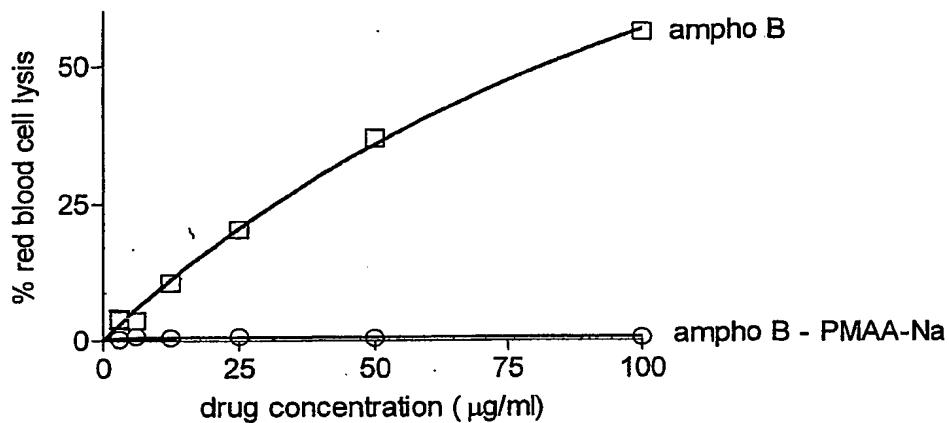


Release of TNF- $\alpha$  from human peritoneal macrophages by PMAA-Na (500  $\mu\text{g/ml}$ ) but not by commercially available PMAA-Na (500  $\mu\text{g/ml}$ ) after 36 h with cells from a single donor B

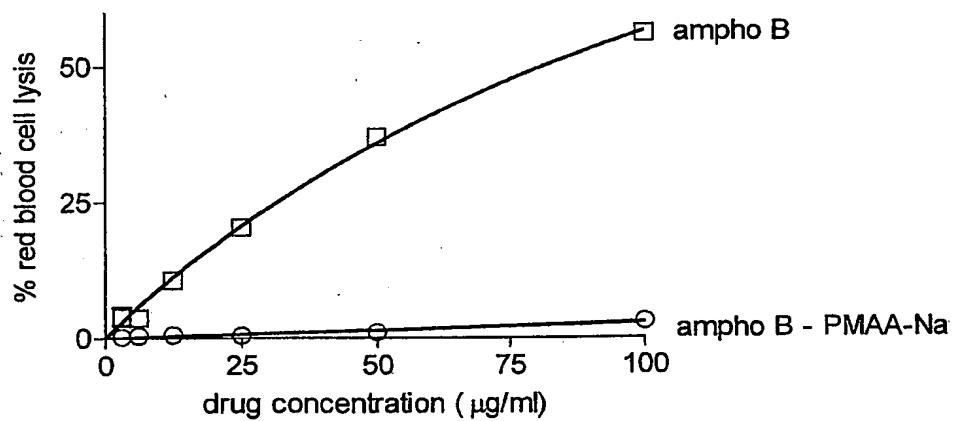


**Figure 10:**

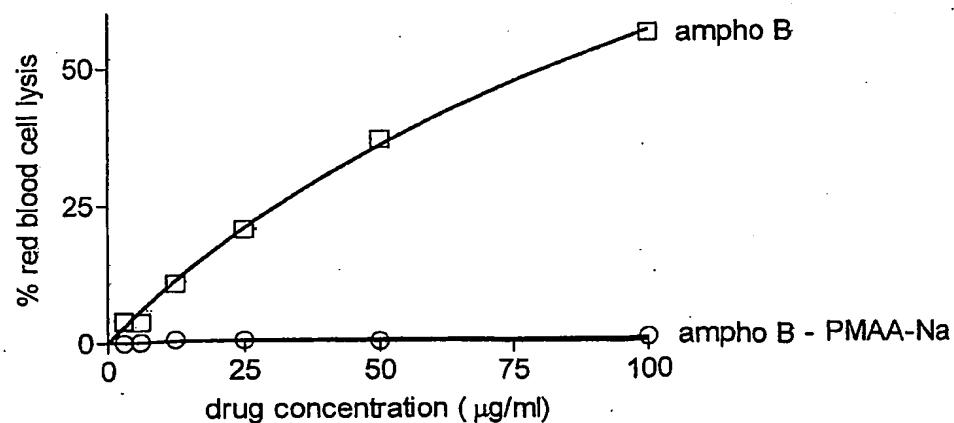
Red blood cell lysis in donor A after a 1 hour incubation in RPMI



Red cell lysis in donor B after a 1 hour incubation in RPMI



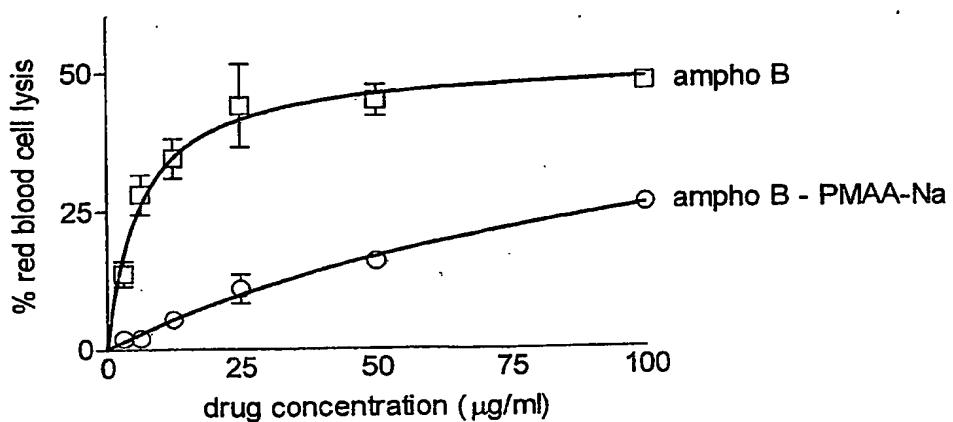
Red blood cell lysis in donor C after a 1 hour incubation in RPMI



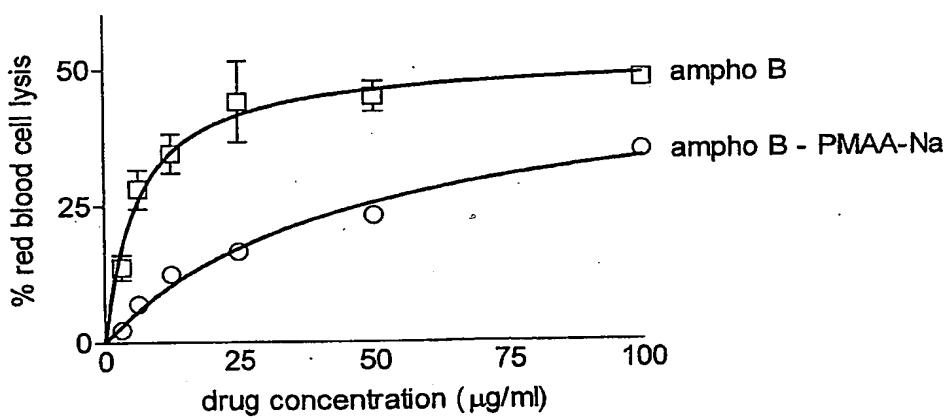
11/26

Figure 11:

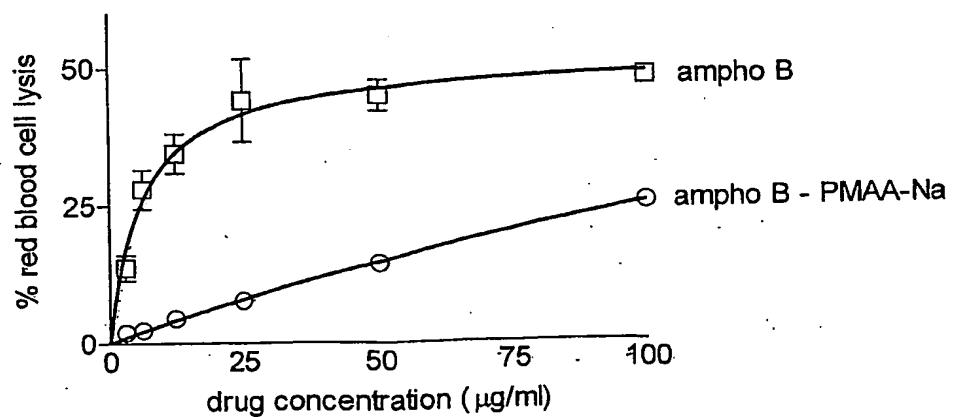
Red blood cell lysis in donor A after a 6 hour incubation in RPMI



Red blood cell lysis in donor B after a 6 hour incubation in RPMI

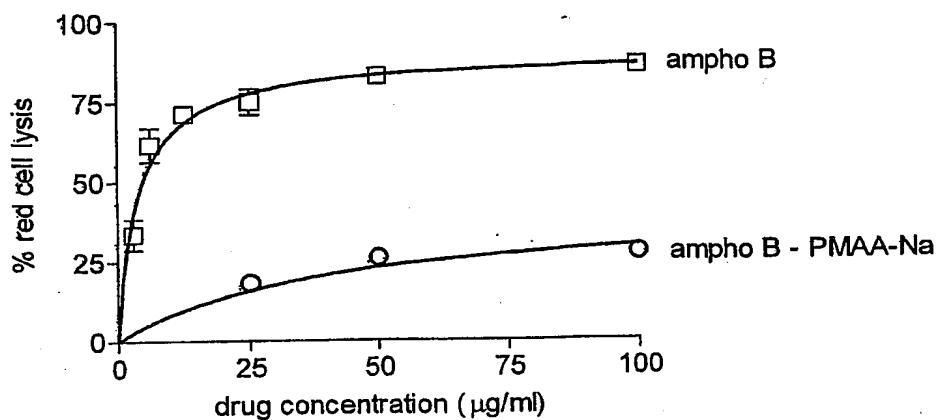


Red cell lysis in donor C after a 6 hour incubation in RPMI

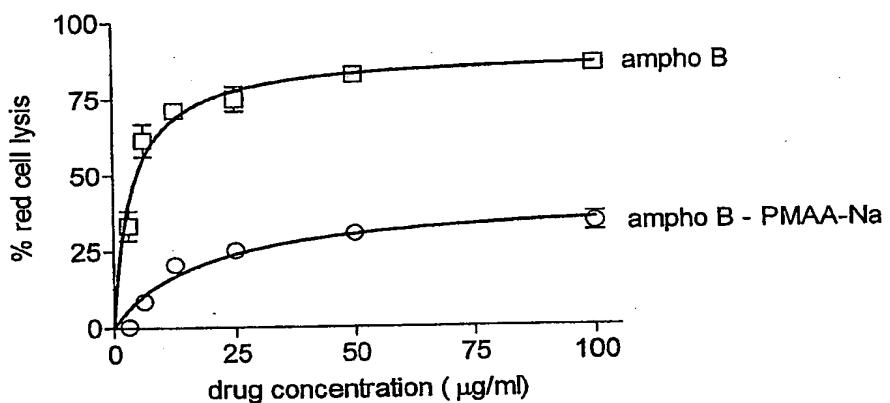


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**Figure 12:**

Red blood cell lysis in donor A after a 24 hour incubation in RPMI



Red cell lysis in donor C after a 24 hour incubation in RPMI



Red blood cell lysis in donor C after a 24 hour incubation in RPMI

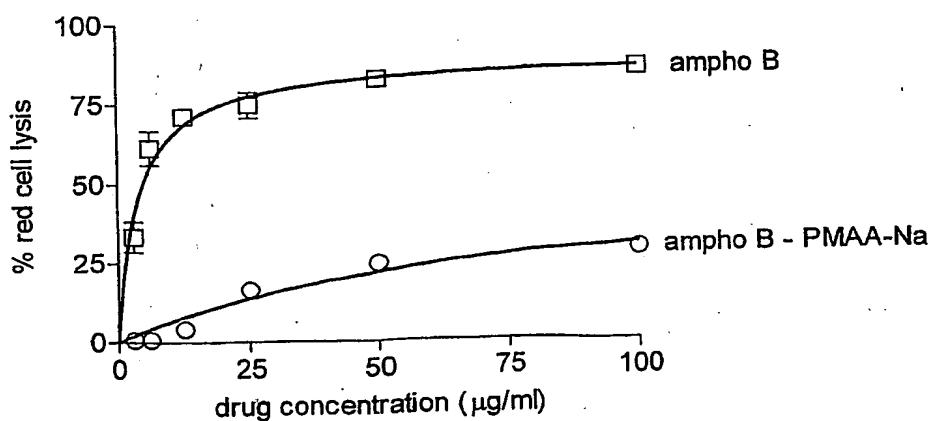
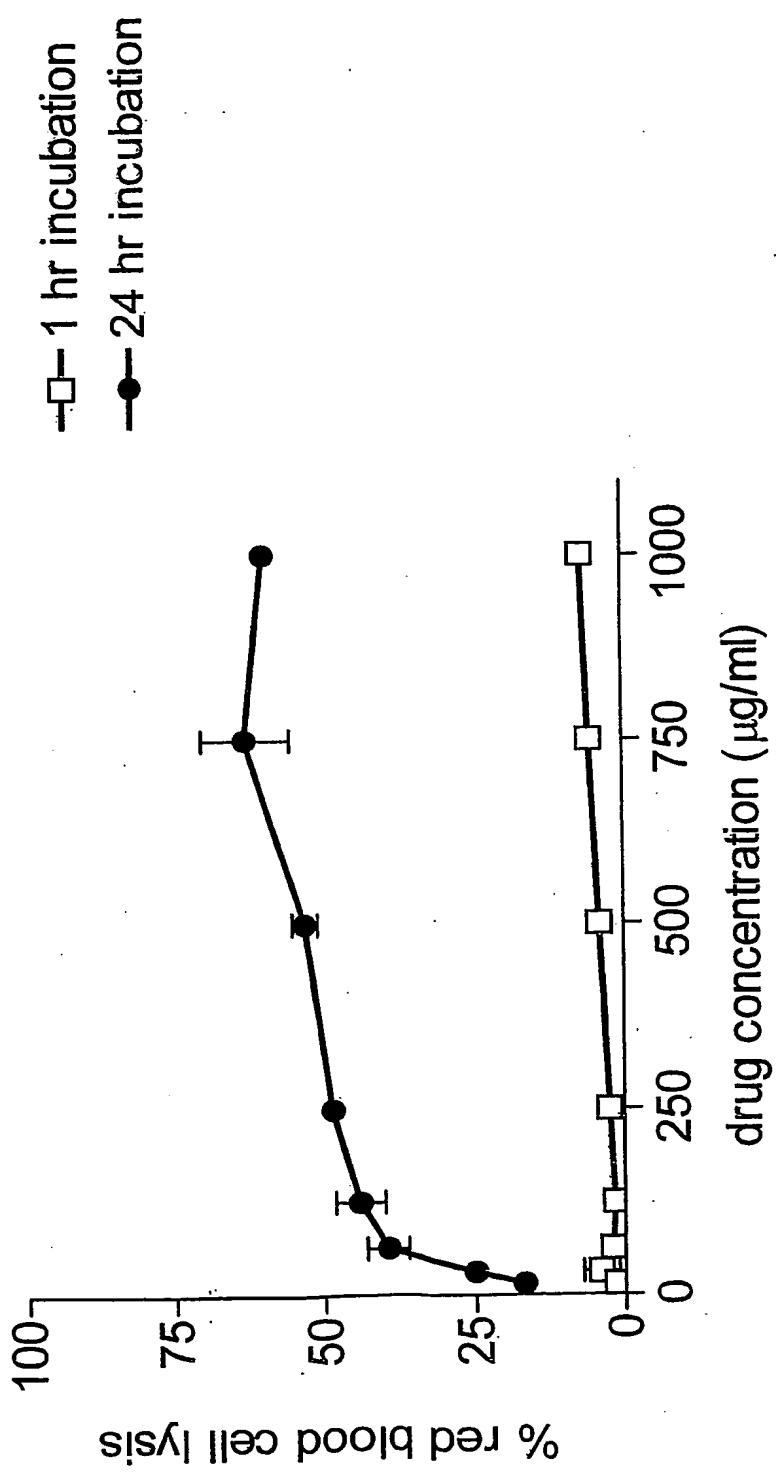


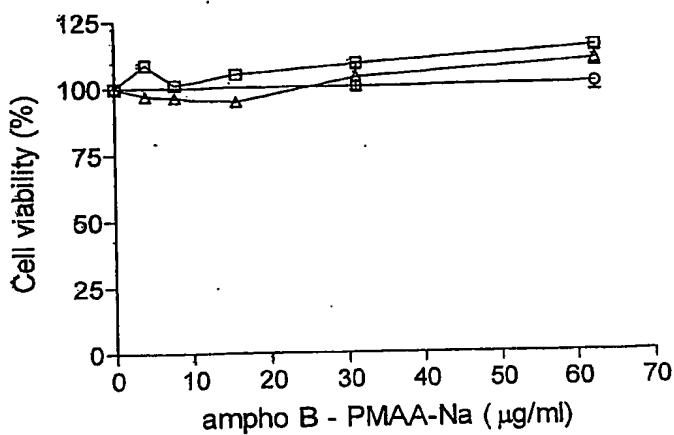
Figure 13:

Red blood cell lysis in single donor after incubation with  
the amphotericin B - PMAA-Na preparation in RPMI

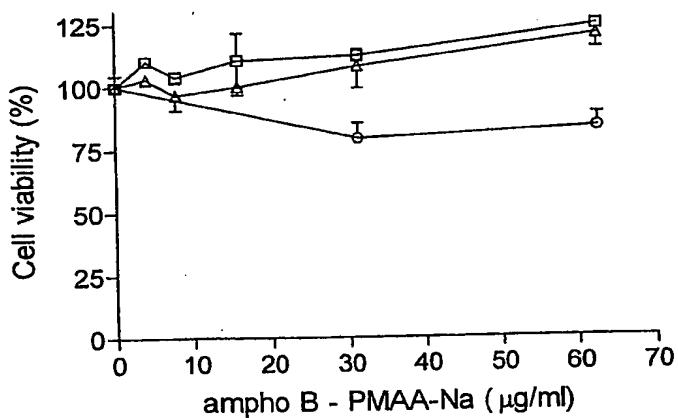


**Figure 14:**

Lack of toxicity of the amphotericin B - PMAA-Na preparation  
after 1 day of culture with PBMN cells (n=3)



Lack of toxicity of the amphotericin B - PMAA-Na preparation  
after 2 days of culture with PBMN cells (n=3)



Lack of the toxicity of amphotericin B - PMAA-Na preparation  
after 6 days of culture with PBMN cells (n=2)

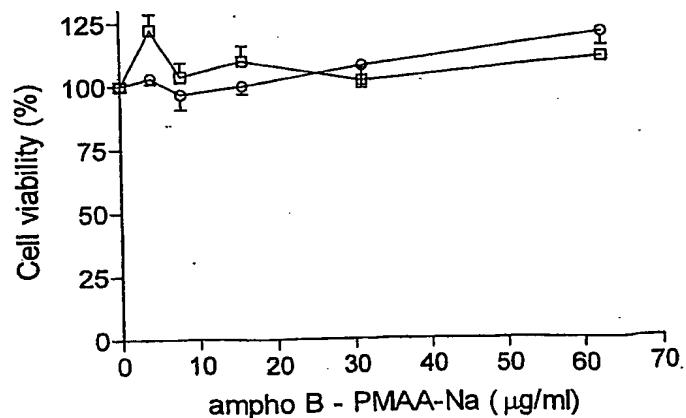
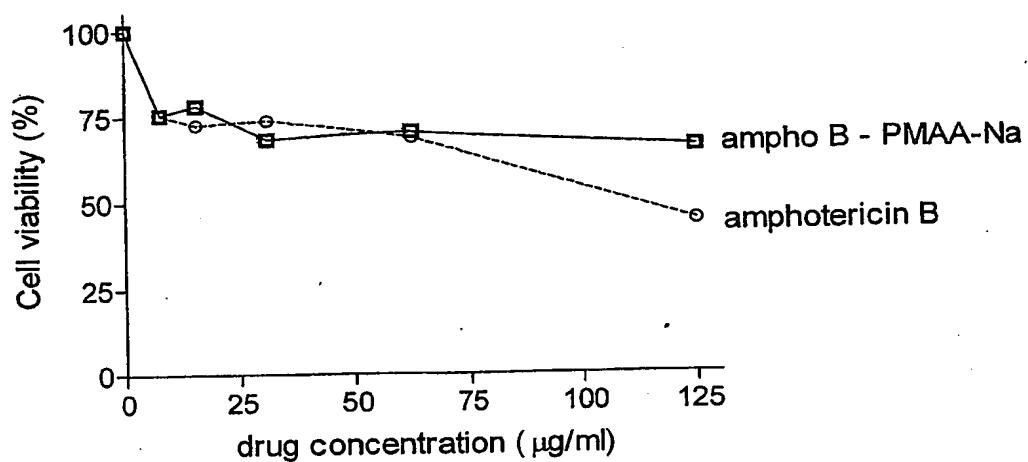
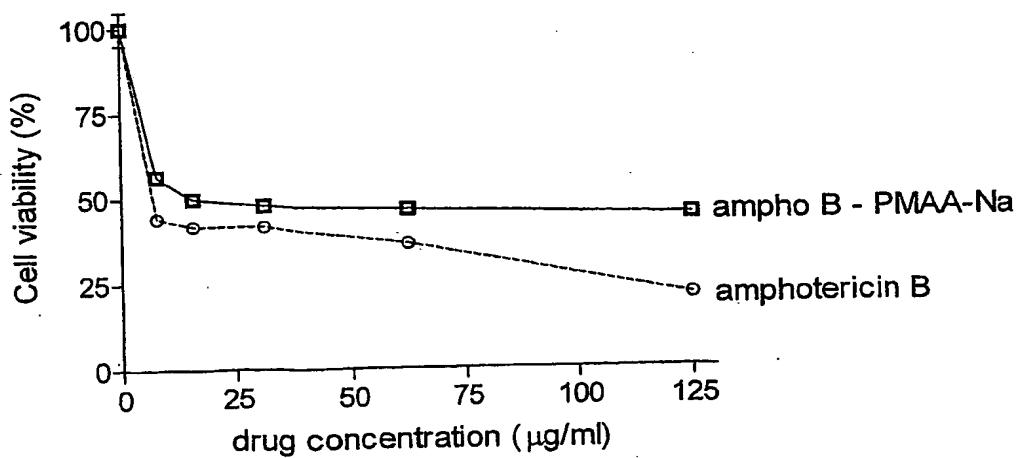


figure 15:

Toxicity of the amphotericin B - PMAA-Na preparation after 2 days of culture with monocyte derived macrophages



Toxicity of the amphotericin B - PMAA-Na preparation after 3 days of culture with monocyte derived macrophages



Viability of *Leishmania mexicana* promastigotes after 2 days

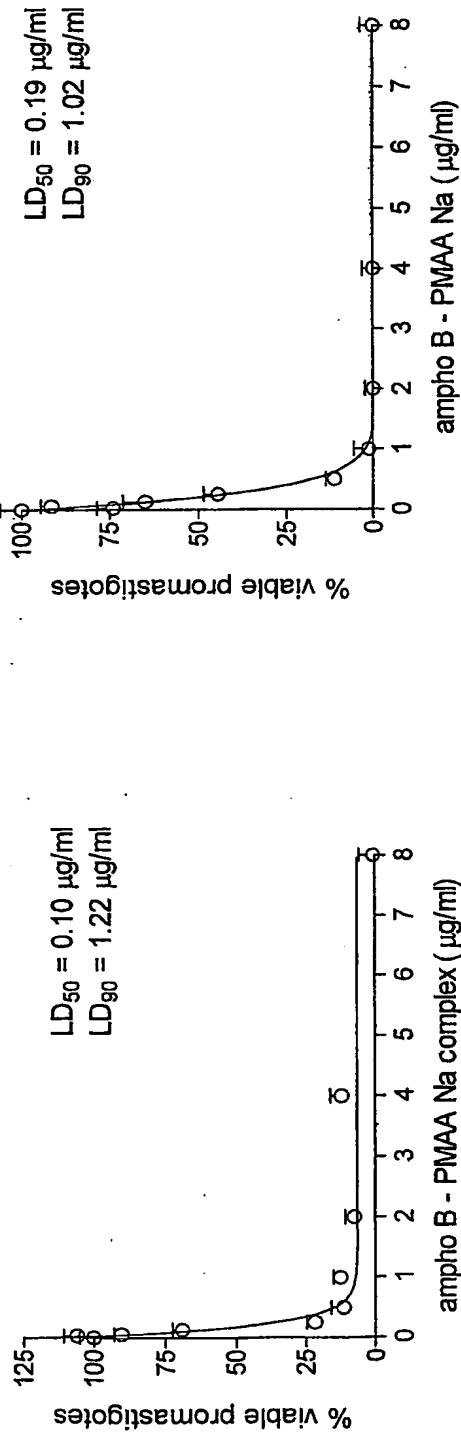
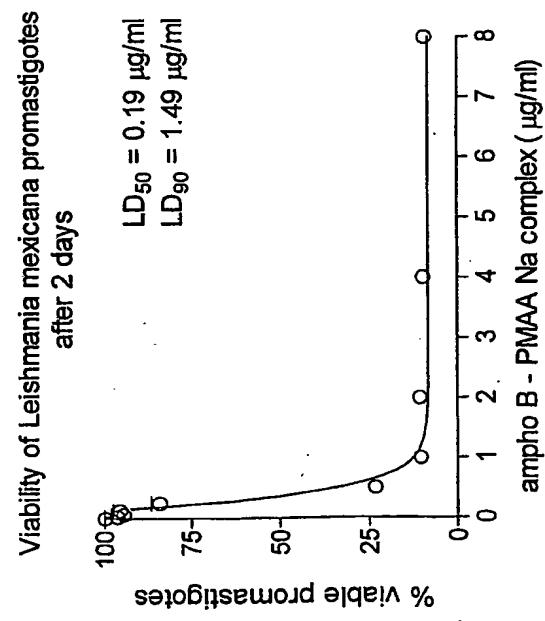
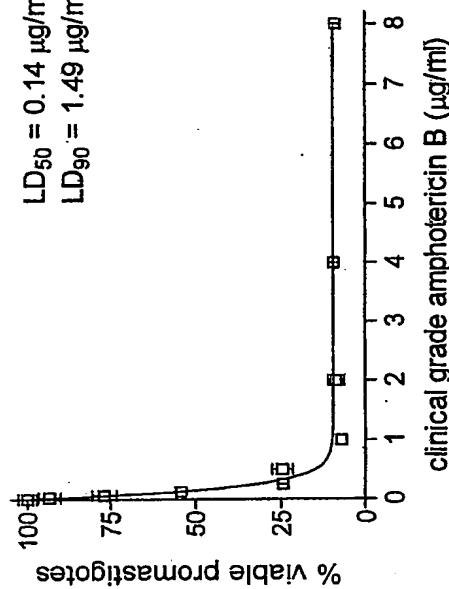


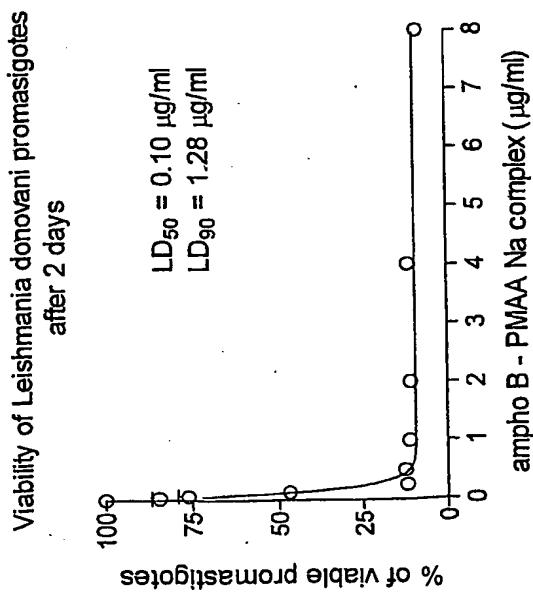
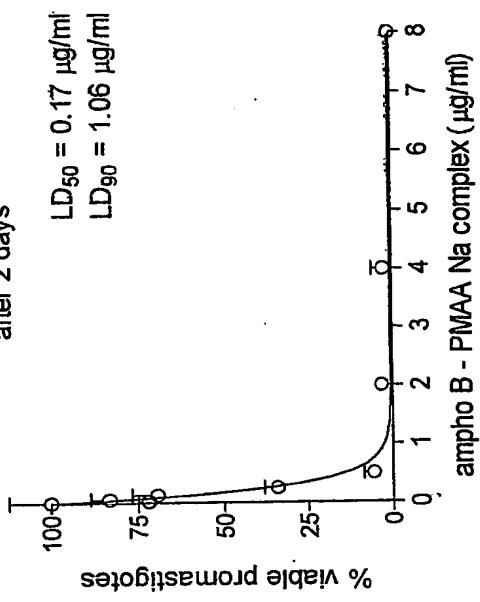
Figure 16:

Viability of *Leishmania mexicana* promastigotes after 2 days



Viability of *Leishmania mexicana* promastigotes after 2 days with clinical grade amphotericin B



**Figure 17:****Viability of Leishmania donovani promastigotes after 2 days****Viability of Leishmania donovani promastigotes after 2 days**

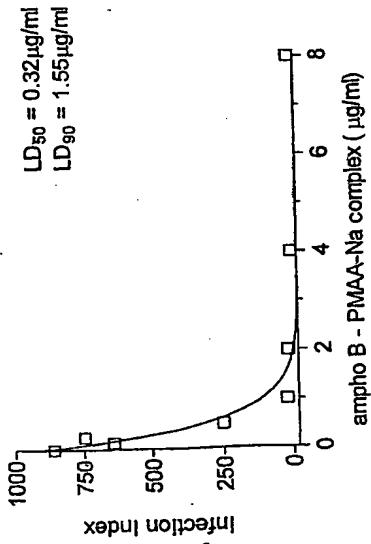
ampho B - PMAA Na (μg/ml)	% of viable promastigotes
0	100
0.1	100
0.2	100
0.5	100
1.0	100
1.5	100
2.0	100
2.5	100
3.0	100
4.0	100
5.0	100
6.0	100
7.0	100
8.0	100

**Viability of Leishmania donovani promastigotes after 2 days with clinical grade amphotericin B**

clinical grade amphotericin B (μg/ml)	% viable promastigotes
0	100
0.1	100
0.2	100
0.5	100
1.0	100
1.5	100
2.0	100
2.5	100
3.0	100
4.0	100
5.0	100
6.0	100
7.0	100
8.0	100

**Viability of Leishmania donovani promastigotes after 2 days with clinical grade amphotericin B**

Inhibition of intracellular Leishmania mexicana amastigote growth by the amphotericin B - PMAA-Na preparation in human monocyte derived macrophages



Inhibition of intracellular Leishmania mexicana amastigote growth by the amphotericin B - PMAA-Na preparation in human monocyte derived macrophages

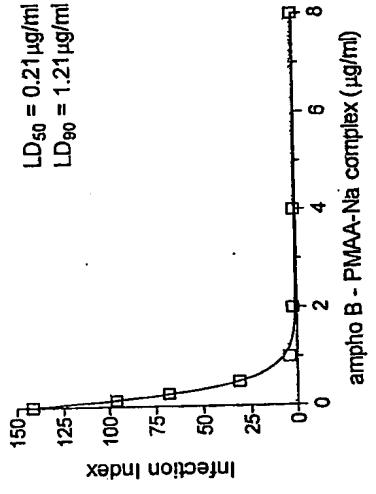
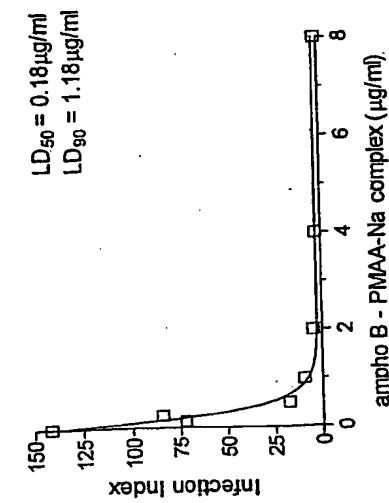
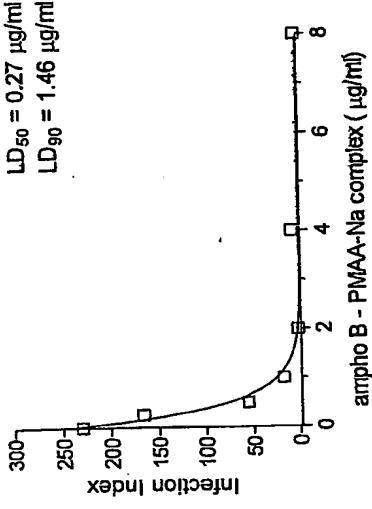


Figure 18:

Inhibition of intracellular Leishmania mexicana amastigote growth by the amphotericin B - PMAA-Na preparation in human monocyte derived macrophages



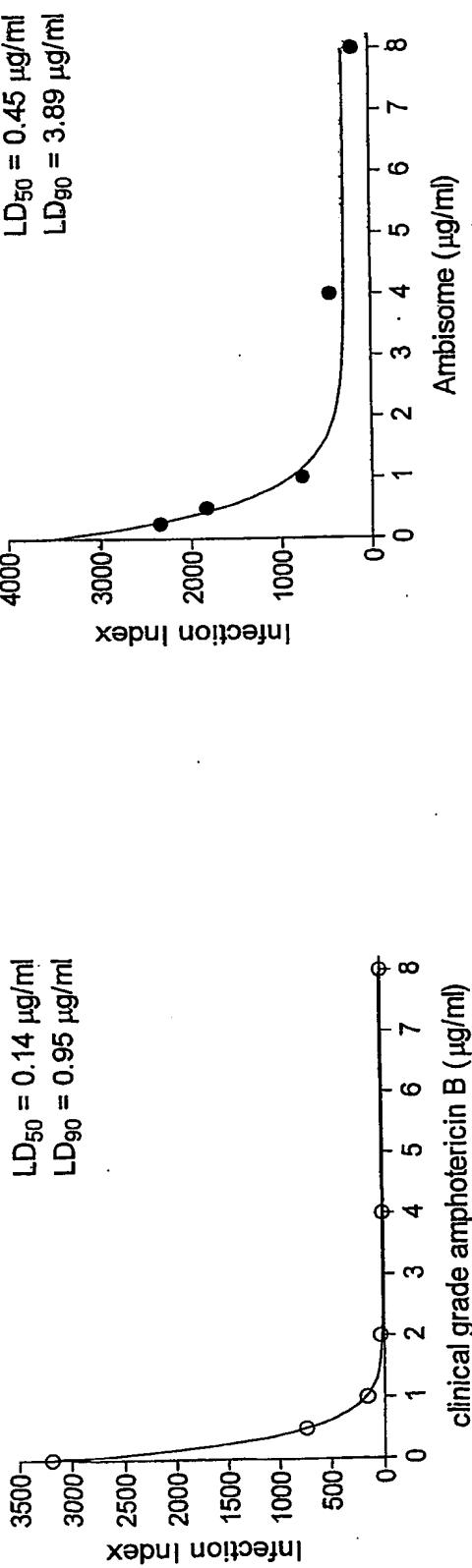
Inhibition of intracellular Leishmania mexicana amastigote growth by the amphotericin B - PMAA-Na preparation in human monocyte derived macrophages



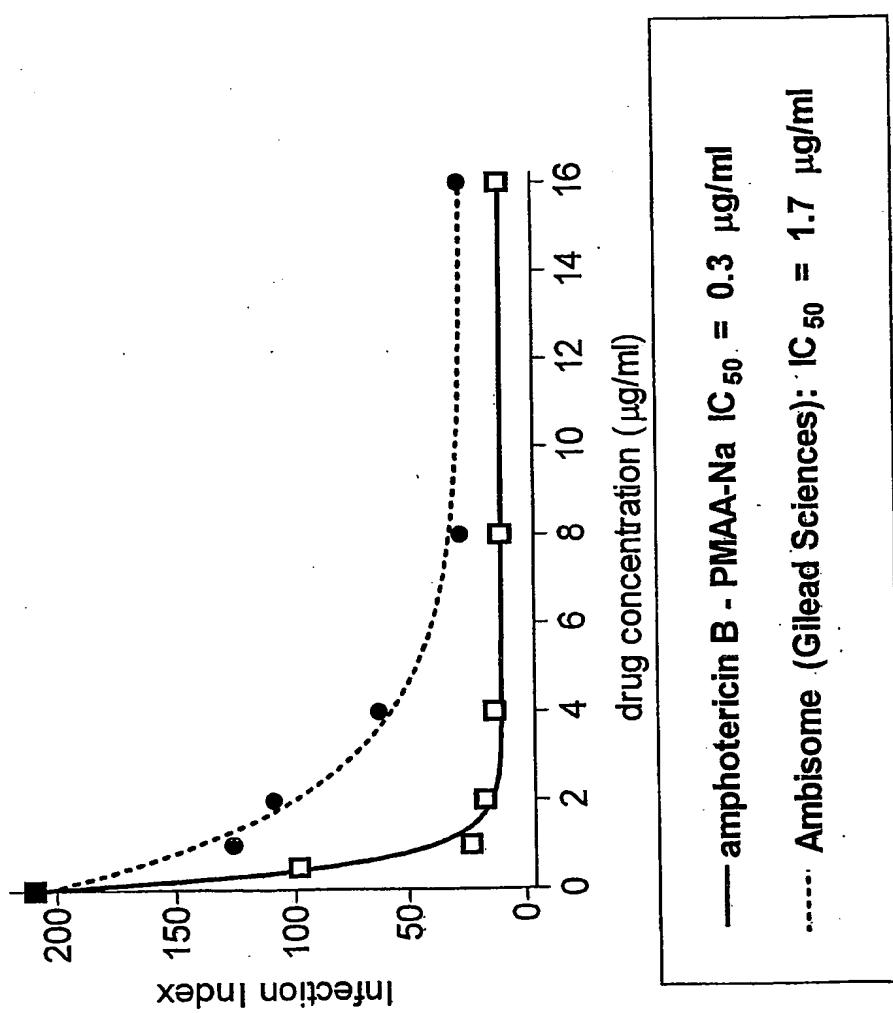
**Figure 19:**

**Inhibition of intracellular *Leishmania mexicana* amastigote growth by clinical grade amphotericin B in human monocyte derived macrophages**

**Inhibition of intracellular *Leishmania mexicana* amastigote growth by Ambisome (Gilead Sciences) in human monocyte derived macrophages**

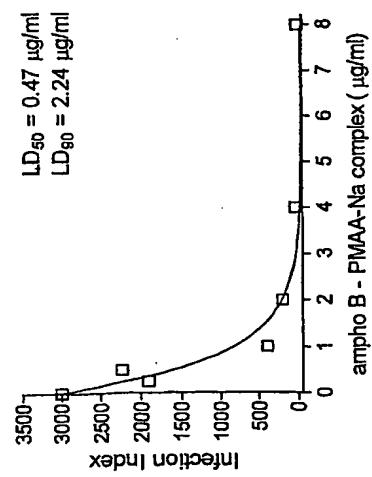


**Figure 20:**  
**Inhibition of intracellular *Leishmania mexicana* amastigote growth in human macrophages  
 by amphotericin B - PMAA-Na compared to AmBisome**

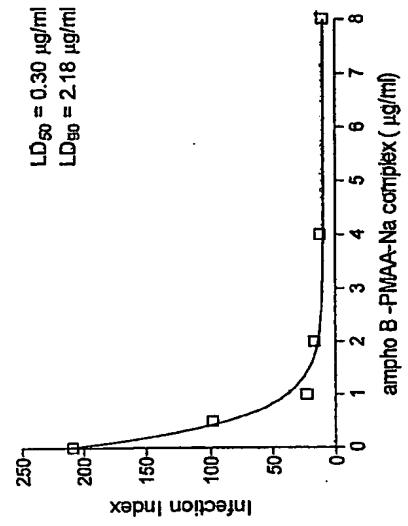


**Figure 21:**

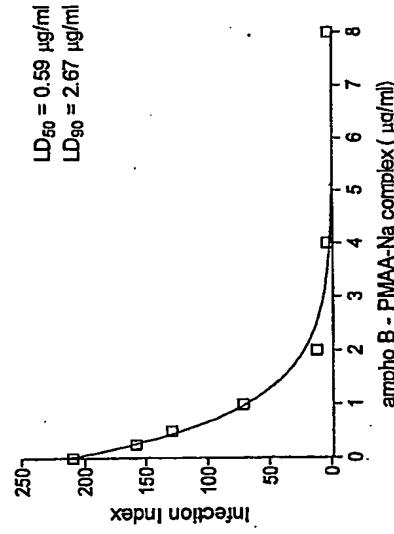
**Inhibition of intracellular *Leishmania donovani* amastigote growth by the amphotericin B-PMAA-Na preparation in human monocyte derived macrophages**



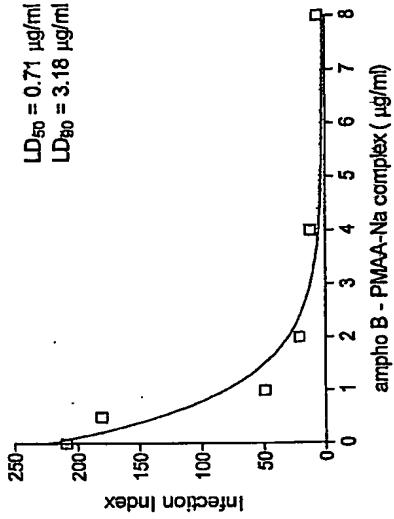
**Inhibition of intracellular *Leishmania donovani* amastigote growth by the amphotericin B-PMAA-Na preparation in human monocyte derived macrophages**



**Inhibition of intracellular *Leishmania donovani* amastigote growth by the amphotericin B-PMAA-Na preparation in human monocyte derived macrophages**



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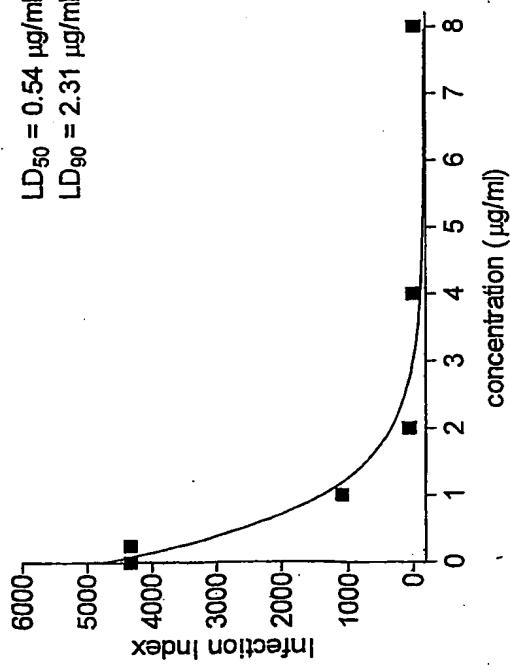
$LD_{50} = 0.30 \mu\text{g/ml}$   
 $LD_{90} = 2.18 \mu\text{g/ml}$

$LD_{50} = 0.71 \mu\text{g/ml}$   
 $LD_{90} = 3.18 \mu\text{g/ml}$

**Figure 22:**

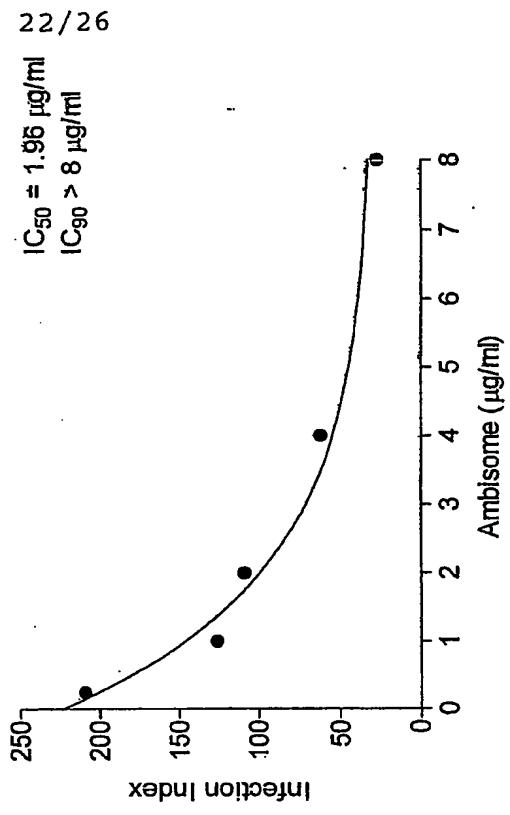
(a)

Inhibition of intracellular *Leishmania donovani* amastigote growth by clinical grade amphotericin B in human monocyte derived macrophages

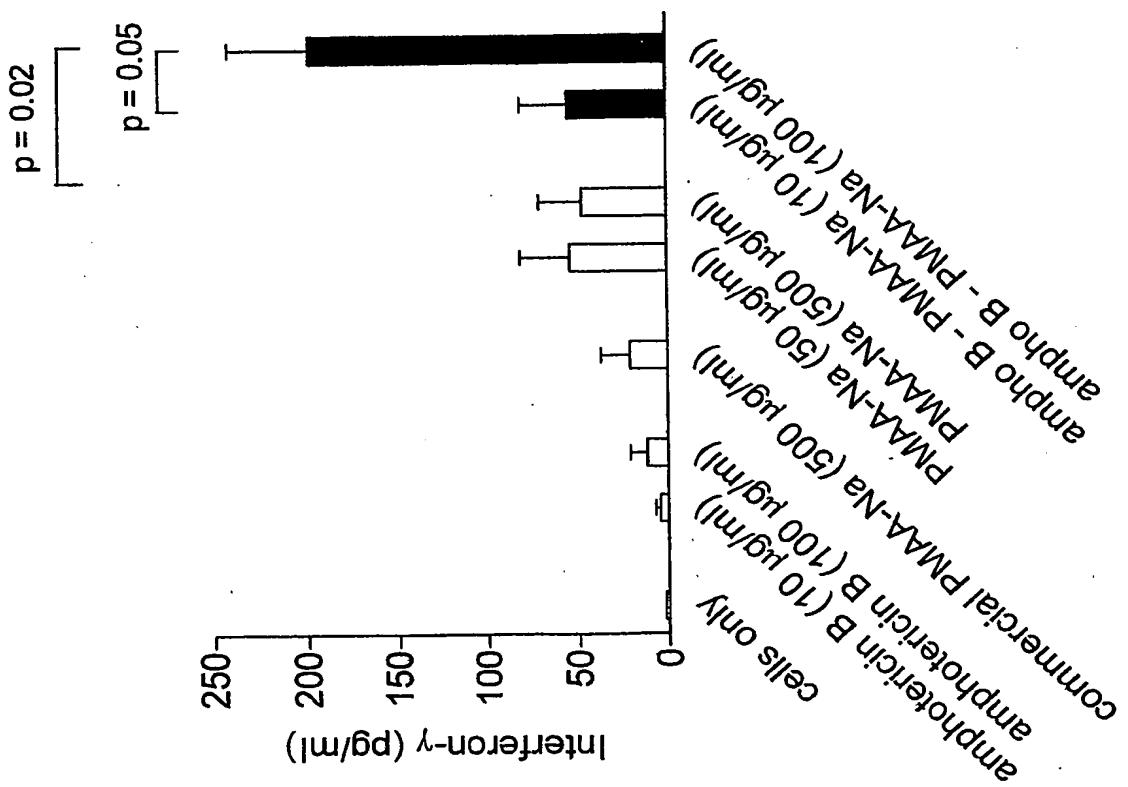


(b)

Inhibition of intracellular *Leishmania donovani* amastigote growth by Ambisome (Gilead Sciences) in human monocyte derived macrophages

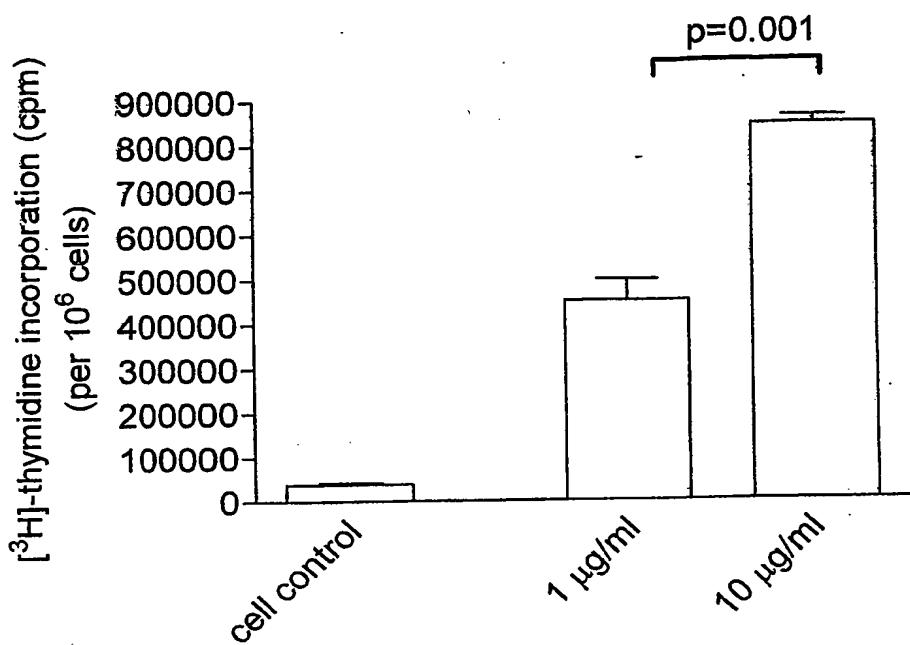


**Figure 23:** Release of interferon- $\gamma$  from human peritoneal macrophages after culture with different compounds for 24 h

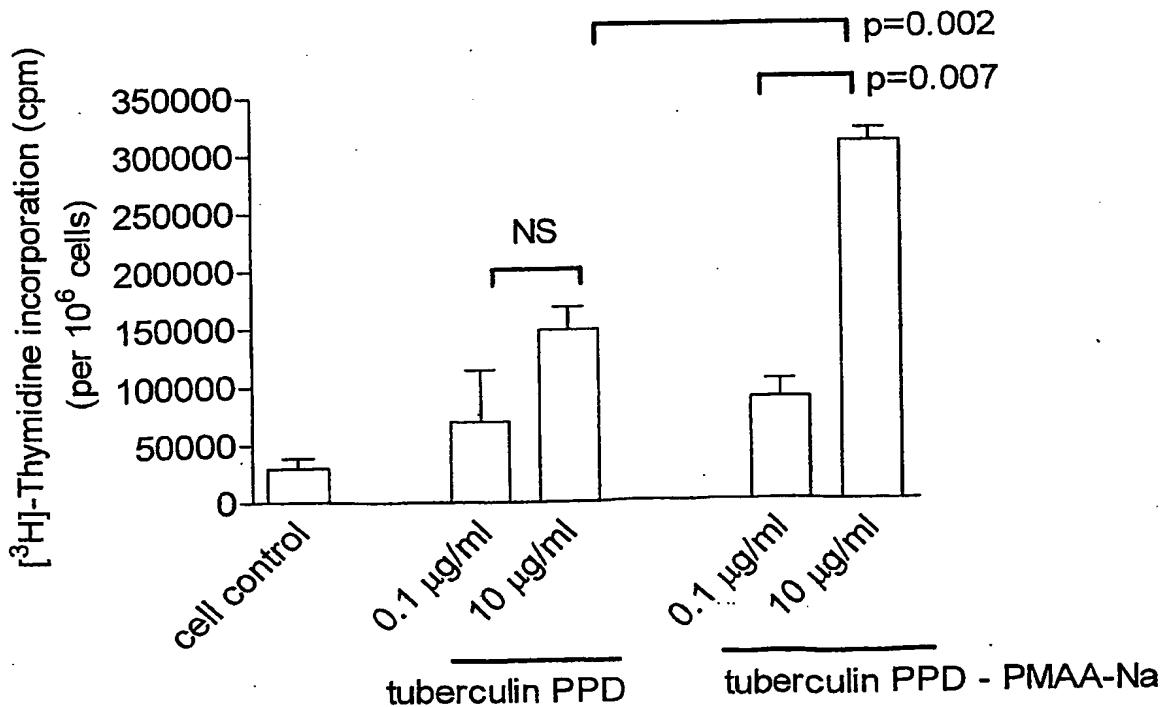


**Figure 24:**

(a) PBMC proliferation after 6 days incubation of the cells of donor A with the tuberculin PPD - PMAA-Na preparation



(b) PBMC proliferation after 5 days stimulation with antigen of the cells from donor B



IFN- $\gamma$  production by human PBMCs  
that were stimulated with antigen from donor A for 24 h

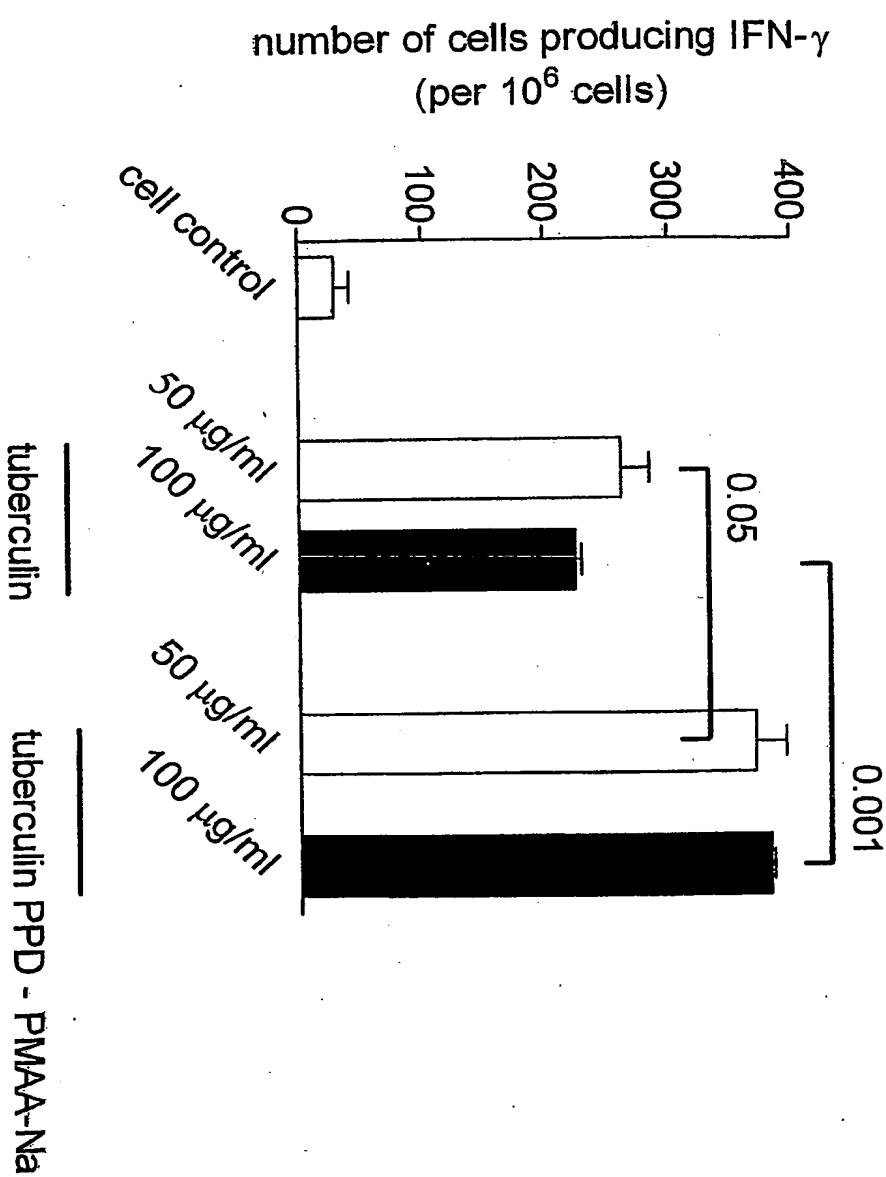
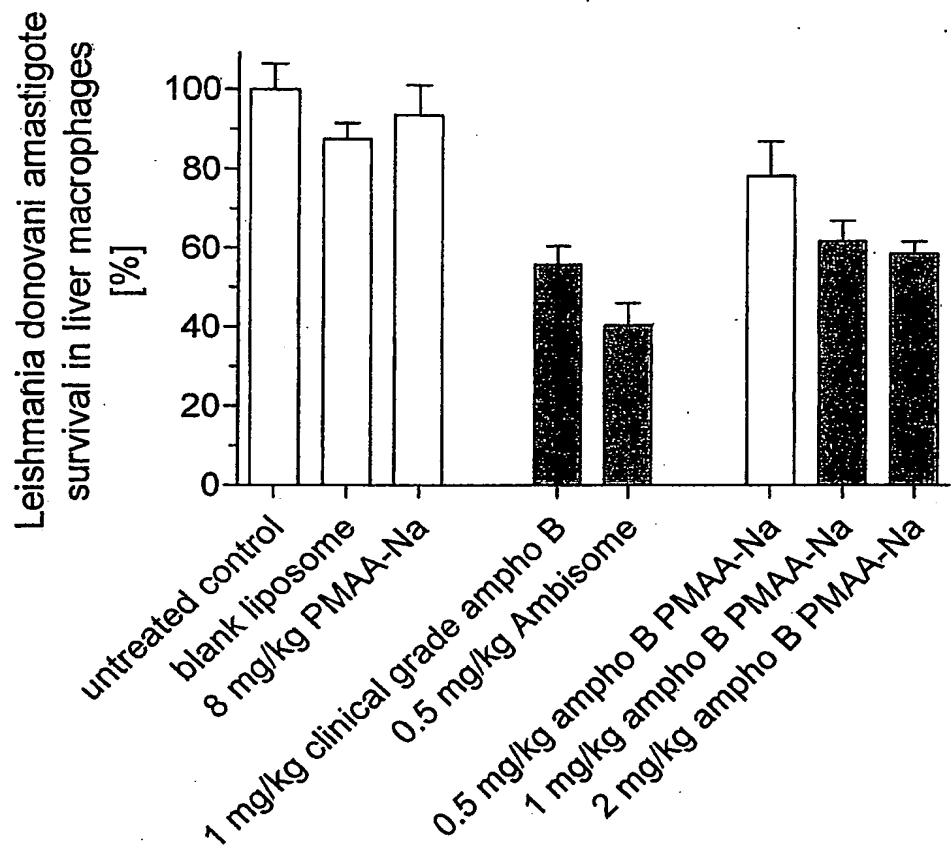


Figure 25:

Survival of *L. donovani* amastigotes in mouse liver macrophages after intravenous treatment with several different preparations of amphotericin B



From the INTERNATIONAL BUREAU

**PCT**

**NOTIFICATION CONCERNING  
SUBMISSION OR TRANSMITTAL  
OF PRIORITY DOCUMENT**

(PCT Administrative Instructions, Section 411)

Date of mailing (day/month/year) <b>21 February 2005 (21.02.2005)</b>	To:  SILVESTON, Judith Abel & Imray 20 Red Lion Street London WC1R 4PQ ROYAUME-UNI
Applicant's or agent's file reference <b>9866/JSpn</b>	<b>IMPORTANT NOTIFICATION</b>
International application No. <b>PCT/GB05/000039</b>	International filing date (day/month/year) <b>07 January 2005 (07.01.2005)</b>
International publication date (day/month/year)	Priority date (day/month/year) <b>07 January 2004 (07.01.2004)</b>
Applicant  <b>POLYTHERICS LIMITED et al</b>	

1. By means of this Form, which replaces any previously issued notification concerning submission or transmittal of priority documents, the applicant is hereby notified of the date of receipt by the International Bureau of the priority document(s) relating to all earlier application(s) whose priority is claimed. Unless otherwise indicated by the letters "NR", in the right-hand column or by an asterisk appearing next to a date of receipt, the priority document concerned was submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b).
2. *(If applicable)* The letters "NR" appearing in the right-hand column denote a priority document which, on the date of mailing of this Form, had not yet been received by the International Bureau under Rule 17.1(a) or (b). Where, under Rule 17.1(a), the priority document must be submitted by the applicant to the receiving Office or the International Bureau, but the applicant fails to submit the priority document within the applicable time limit under that Rule, the attention of the applicant is directed to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.
3. *(If applicable)* An asterisk (\*) appearing next to a date of receipt, in the right-hand column, denotes a priority document submitted or transmitted to the International Bureau but not in compliance with Rule 17.1(a) or (b) (the priority document was received after the time limit prescribed in Rule 17.1(a) or the request to prepare and transmit the priority document was submitted to the receiving Office after the applicable time limit under Rule 17.1(b)). Even though the priority document was not furnished in compliance with Rule 17.1(a) or (b), the International Bureau will nevertheless transmit a copy of the document to the designated Offices, for their consideration. In case such a copy is not accepted by the designated Office as the priority document, Rule 17.1(c) provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

<u>Priority date</u>	<u>Priority application No.</u>	<u>Country or regional Office or PCT receiving Office</u>	<u>Date of receipt of priority document</u>
07 January 2004 (07.01.2004)	0400264.8	GB	17 February 2005 (17.02.2005)

4)

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland  Facsimile No. +41 22 740 14 35	Authorized officer  <b>Mulhausen Dorothee</b>  Facsimile No. +41 22 338 87 40 Telephone No. +41 22 338 9672
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